IMMUNOLOCALIZATION AND *IN VIVO* FUNCTIONAL ANALYSIS BY RNAI OF THE *AEDES* KININ RECEPTOR IN FEMALE MOSQUITOES OF *AEDES AEGYPTI* (L.) (DIPTERA, CULICIDAE)

A Thesis

by

CYMON NICHOLE KERSCH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2011

Major Subject: Entomology

Immunolocalization and *in vivo* Functional Analysis by RNAi

of the *Aedes* Kinin Receptor in Female

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Approved by:

Chair of Committee,	Patricia V. Pietrantonio
Committee Members,	Albert Mulenga
	Karen Snowden
Head of Department,	David Ragsdale

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ABSTRACT

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(December 2011)

Cymon Nichole Kersch, B.S., University of Puget Sound

Chair of Advisory Committee: Dr. Patricia V. Pietrantonio

The evolution of the blood feeding adaptation has required precise coordination of multiple physiological processes in the insect, such as reproduction, behavior, digestion and diuresis. These processes are under careful synchronous hormonal control. For rapid excretion, multiple diuretic hormones are known. Although originally described based on their ability to stimulate hindgut contractions, the *Aedes* kinins have been shown to stimulate fluid secretion in female mosquitoes of *Aedes aegypti*. *Aedes* kinins are leucokinin-like neuropeptides released from neurosecretory cells in the brain and abdominal ganglia. They act by binding to the *Aedes* kinin receptor, a G protein-coupled receptor (GPCR). The *Aedes* kinin receptor has been cloned, sequenced, functionally characterized, and immunolocalized to stellate cells in the Malpighian tubules of *Ae. aegypti.* In addition to their myotropic and diuretic roles, leucokinin-like peptides and/or their receptors have been also been discovered in the nervous, digestive, and reproductive systems of other arthropod species. Therefore, the *Aedes* kinins have

the potential to function in several simultaneous physiological processes that are stimulated by blood feeding. This thesis aims to understand better their role in the whole mosquito by investigating the *Aedes* kinin receptor's global expression as well as its *in vivo* contribution to post-prandial diuresis.

Presence of the *Aedes* kinin receptor was investigated in the head, posterior midgut (stomach), hindgut, ovaries, and Malpighian tubules of both non blood-fed and blood-fed females by western blot using anti-receptor antibodies. The receptor was then immunolocalized in the posterior midgut and rectum. Finally, RNAi was employed to knock down kinin receptor expression, followed by measurement of *in vivo* urine excretion post blood feeding in a precision humidity chamber. Transcript and protein knockdown were confirmed by qPCR and immunohistochemistry, respectively.

Results indicate widespread expression of the *Aedes* kinin receptor protein in organs novel for hematophagous insects and demonstrate the receptor's fundamental role in rapid diuresis. These findings strongly point to the *Aedes* kinins as integrative signaling molecules that could coordinate multiple physiological systems. The *Aedes* kinins could therefore have contributed to the success of the blood feeding adapation in mosquitoes.

ACKNOWLEDGEMENTS

I would like to dedicate this thesis to my parents and brother, whose support throughout both my undergraduate and graduate studies has given me the strength, drive, and curiosity to pursue science. Their influence has pushed me to appreciate scientific discovery and the beauty of pursing knowledge, and I sincerely thank them for that.

I would also like to thank my committee chair, Dr. Patricia Pietrantonio, whose influence on my scientific endeavor began long before my graduate studies. The summer between my junior and senior years of undergraduate education at the University of Puget Sound, she accepted me as a member of her laboratory through a Research Experience for Undergraduate (REU) program sponsored by the National Science Foundation. That summer was my first research experience, and her guidance and positive feedback were pivotal to my decision to apply to graduate school and further explore research. After beginning my Master of Science in Dr. Pietrantonio's laboratory, she gave me endless opportunity to excel while learning numerous molecular laboratory techniques and publishing peer reviewed papers. I cannot thank her enough for her constant support.

I am also exceedingly appreciative of the support and guidance of my committee members, Dr. Albert Mulenga and Dr. Karen Snowden. Their encouragement, support and suggestions on my research, multiple recommendation letters, flexibility, and positive feedback were infinitely helpful throughout my Masters. I would also like to thank them for their wonderful courses: medical entomology and biomedical

parasitology, which quickly became two of my favorite courses here at Texas A&M University. I hope to take the material I learned in these classes and apply it to a future career in infectious disease medicine.

Finally, I would like to thank my colleagues, friends, department faculty and staff for making my time at Texas A&M University enjoyable and a memorable experience. My lab members, Hsiao-Ling, “Sunny”, “Andy”, and Brad, guided me through my research, teaching me the expertise they had gained and passing on the “tricks of the trade.” I owe much credit for my research and success with this project to their guidance and assistance in the lab. Thank you all so much!

I also want to extend my gratitude to the Texas A&M University College of Agriculture and Life Sciences for funding the first year of my research, and to Dr. Pietrantonio for her support and funding during my second year.

NOMENCLATURE

*Ae*KR	*Aedes* Kinin Receptor
CDC	Center for Disease Control
DHF	Dengue Haemorrhagic Fever
EGFP	Enhanced Green Fluorescent Protein
GPCR	G Protein-Coupled Receptor
MT	Malpighian Tubules
NBF	Non Blood Fed
PBM	Post Blood Meal
PE	Post Eclosion
qPCR	Relative Quantitative Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
RT	Room Temperature
SAR	Structure Activity Relationship
siRNA	Small Interfering RNAs
TAMU	Texas A&M University
WHO	World Health Organization

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CHAPTER I

INTRODUCTION: THE BLOOD FEEDING ADAPTATION AND THE *AEDES* KININ RECEPTOR

1.1 The mosquito *Aedes aegypti* as a critical pathogen vector in global public health

Improving knowledge of the complex life cycles and biologies of many disease causing pathogens, their vectors, and the definitive human host may help combat many wide-spread diseases. Candidate diseases for complete eradication have been recently discussed (Enserink, 2010). While smallpox and severe acute respiratory syndrome (SARS) are the only diseases to ever be successfully eradicated at a global level, the eradication of both polio and guinea worm disease appear possible within the near future, and lymphatic filariasis, measles, river blindness, and malaria may be promising future candidates for these programs. Interestingly, of the future disease candidates noted, half of their causative pathogens utilize mosquito vectors for transmission, highlighting the unequivocal importance of mosquitoes in global health. Thus, a more detailed understanding of the mosquito life cycle and their physiological processes related to pathogen transmission and survival may be critical for developing the most cost effective, efficient, and successful disease control programs.

This thesis follows the style of Insect Biochemistry and Molecular Biology.

While multitudes of different mosquito species are pathogen vectors, many are difficult to maintain in laboratories or modify and manipulate for experimental procedures. Therefore, *Aedes aegypti*, which are easy to rear in the laboratory and whose genome has been sequenced and annotated, has become a research standard for studying these insects (Clemons et al., 2010). Even the *Bill and Melinda Gates Foundation* funds significant research projects that employ *Ae. aegypti* as a model to better understand the physiology and vectorial capacity of *Anopheline* mosquitoes.

Beyond their use as model organisms, female *Aedes aegypti* mosquitoes are vectors of the causative pathogens for both yellow fever and dengue fever diseases. Worldwide, there are an estimated 200,000 cases of yellow fever per year, resulting in more than 30,000 deaths (WHO, 2010b). Although there is an effective vaccine available, global epidemics have increased in the past two decades due to human population and environmental changes, including large movements of displaced persons by war, increased international travel, and global climate change (Hales et al., 2002; McMichael et al., 2006). In the case of dengue, infection rates have been rising, despite control efforts by both government and private organizations. At present, more than 2.5 billion people are at risk of dengue infection. Multiple infections with different dengue virus serotypes can cause a lethal complication: dengue haemorrhagic fever (DHF). DHF is the leading cause of serious illness and death of children in several Asian countries (WHO, 2010a). Unlike for yellow fever, there is currently no licensed vaccine for dengue. Thus, disease management concentrates on controlling the disease-vectoring mosquito populations.

Ae. aegypti populate a wide range of habitats on almost every continent, making population control difficult. One of the major methods to control mosquito populations is insecticide application. While this approach has been invaluable for mosquito control, many insecticides are non-selective and/or accumulate in the environment with numerous harmful side effects. Thus, the number of legally available insecticides has declined due to increasingly stringent laws promoting environmental health and sustainability. Furthermore, there is growing resistance to many insecticide chemical classes. For instance, numerous carbamates and organophosphates, such as the commonly used Malathion, are becoming less effective due to insect resistance, which is diminishing their importance in mosquito control (Rodríguez et al., 2007). Given the decrease in effective and available insecticides, chemicals with new modes-of-action are in high demand.

1.2 Potential solutions: research on novel molecular targets for mosquito control

Current research in insect endocrinology is focused on the search for new molecular targets for novel insecticides. Particularly, identifying and characterizing new targets involved in functions critical for disease transmission is an attractive direction for research (Gäde, 2004; van Hiel et al., 2010). For instance, since hematophagous insects such as mosquitoes transmit pathogens to a susceptible vertebrate host during blood feeding, hormones and proteins that are important for proper blood feeding and the

related physiological process (such as digestion, osmoregulation, excretion, and oogenesis) are candidate targets.

The evolution of blood feeding in mosquitoes, possibly due to a switch from ancestral entomophagy to hematophagy, required multiple adaptations (Klowden, 1995; Waage, 1979). These adaptations can be broken down into three categories: morphological, physiological, and behavioral. Morphological adaptations include piercing mouthparts that enable females to acquire blood. Physiological adaptations include the presence of proteolytic enzymes for digestion, rapid diuresis post blood feeding to void excess fluid and ions, and the acquisition of blood meal proteins for yolk and egg development. Behavioral modifications are separated into two groups: (1) host-seeking behaviors, which allow the female to distinguish and locate hosts through visual cues, carbon dioxide, heat, lactic acid and other unknown olfactory cues, and (2) blood feeding behavior, which permit the mosquito to recognize close-range stimuli from the host, puncture the skin, secrete anti-coagulants, stop feeding when appropriately full and rest until oviposition. Particularly intriguing molecular targets for current and future research are hormones and their receptors that may simultaneously contribute to the success of multiple components of the blood feeding adaptations such as feeding behavior, blood meal digestions and excretion, omoregulation for hemolymph homeostasis, and oogenesis.

1.3 Hematophagy and oogenesis

The blood meal is essential for females to acquire proteins for egg production making it a critical step in reproduction, and thus, species survival. When female mosquitoes emerge as adults their ovaries contain only one follicle per ovariole and the germarium (Swevers et al., 2005). After signaling by 20-hydroxyecdysone and adult juvenile hormone (JH), the ovary progresses to a "resting stage." At this stage, usually 72 hours post eclosion, the female seeks a protein-rich blood meal to initiate vitellogenesis (Fallon et al., 1974; Flanagan and Hagedorn, 1977; Hagedorn, 1974; Lea, 1972; Simonet et al., 2004). Dipteran females, such as mosquitoes, have polytrophic meroistic ovaries, meaning there are nurse cells associated with each oocyte, the developing egg (Nation, 2008). After the blood meal has initiated the vitellogenic stage of ovary and egg development, hormonal control drives development in the ovary. Many of these hormones and their functional roles in oogenesis (egg development) have been elucidated, such as the roles of JH, corpora cardiaca stimulating factor (CCSF), egg development neurohormone (EDNH) also called ovarian ecdysteroidogenic hormone I (OEH), 20-hydroxyecdysone, and trypsin modulating oostatic factor (TMOF) more simply known as oostatic hormone (OSH) (Klowden, 1987; Klowden, 1997; Nation, 2008). However, there are still "black boxes" in our overall understanding of ovary development, leaving the possibility of further hormones assisting to regulate the process very likely.

In addition to gaining a more complete view of the hormones regulating oogenesis, research studying hormonal control of mosquito reproduction is essential for future vector control programs. *Ae. aegypti* are anautogenous, meaning they require a blood meal for all egg production. In contrast, several other mosquito species are autogenous and can lay their first batch of eggs utilizing protein reserves from larval stages. Given that *Ae. aegypti* require a blood meal for each subsequent batch of eggs to develop, they feed frequently on different hosts making them efficient disease vectors (Zhu et al., 2003). When fully engorged, females ingest more than 10 times their hemolymph volume in blood. As the blood meal is hypoosmotic with respect to the hemolymph, has a different ion composition, and adds weight hindering flight ability, the meal conveys a considerable fitness cost (Adams, 1999).

1.4 Digestion and excretion of a blood meal

To overcome the potential problems caused by the large blood meal that could negatively affect mosquito survival, females rapidly excrete fluid and ions post feeding (postprandial diuresis). In some mosquito species diuresis begins before feeding has ceased (Stobbart, 1977). Postprandial diuresis can be distinguished into three phases: peak, post peak, and late (Bradley, 1987; Williams et al., 1983). The peak phase lasts approximately 15 minutes immediately following the meal and during this phase the urine excretion rates average 54 nl/min. The following 45 minutes (15 - 60 min post blood meal) constitutes the post peak phase with a fluid excretion rate of 11 nl/min.

During this first hour post blood feeding the female excretes nearly 40% water, sodium, and chloride from the ingested blood meal (Stobbart, 1977; Williams et al., 1983). Finally, 60 – 120 minutes post blood meal is the late phase where the urine excretion rate slows to an average of 3 nl/min (Beyenbach, 2003a). Although in reality these three phases operate on a continuum, studying them allows for research targeted at understanding sequential hormonal triggers and regulators during postprandial diuresis.

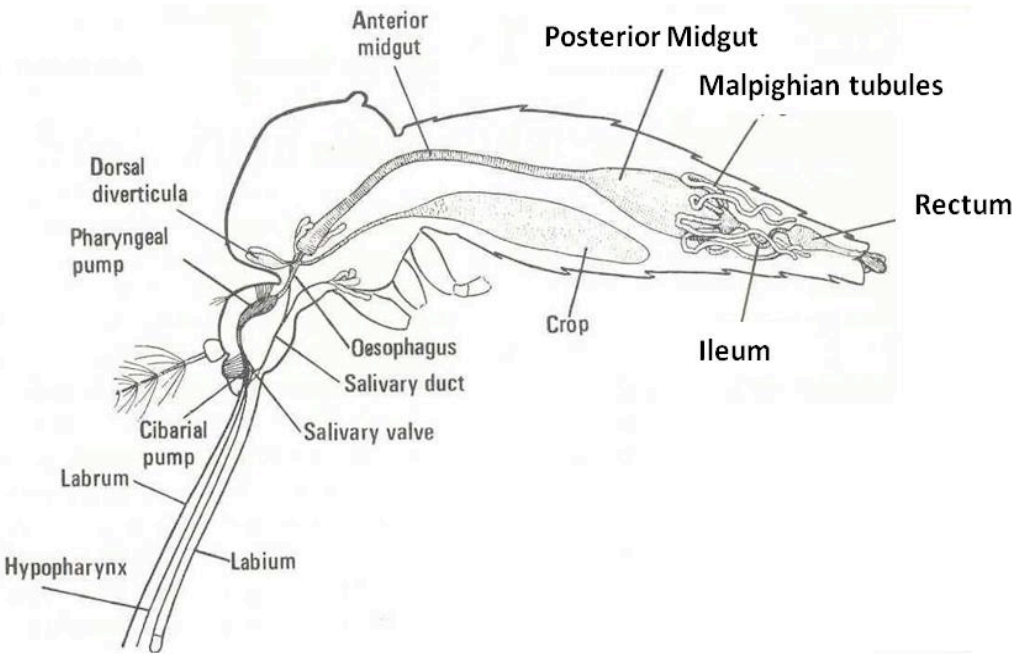


Figure 1. The mosquito digestive system. Key organs in the mosquito digestive system include the midgut, Malpighian tubules, ileum and rectum (adapted from Clements, 1992).

For rapid diuresis to occur, the blood meal must be quickly digested and processed through a series of digestive and excretory organs (Figure 1). Initially, the blood meal passes through the foregut, which is modified in mosquitoes to tolerate large

liquid meals (Bradley, 1987). However, as the foregut is of ectodermal origin there is a cuticular lining and no ion exchange occurs between the food bolus and hemolymph in this region of the alimentary canal.

Next, in the midgut, the large blood bolus distends the abdomen, triggering stretch receptors. Activating the stretch receptors likely plays a role in signaling rapid diuresis. Stretch receptors also function in behavior relating to "fullness" and signaling the insect to stop feeding (Gwadz, 1969). In the midgut, hematin and other proteins are stored, digested, then passed through the pylorus and into the hindgut for excretion, while water and ions are absorbed from the midgut lumen and transported to the hemolymph (van Handel and Klowden, 1996). Regulation of digestion and exchange of ions and fluid across the midgut epithelium is coordinated by both hormones and innervation. In the posterior midgut where the meal is stored and digested, approximately 500 endocrine cells have been identified in *Ae. aegypti* (Brown et al., 1985; Graf et al., 1986). These endocrine cells are small, thin and cone-shaped and are dispersed among larger columnar epithelial cells. They can either release their contents into the gut lumen or into the hemolymph, based on each specific endocrine cell type. While there is gene expression and immunohistologic data showing that numerous peptides including locustatachykinins (LTKs) and tachykinin-related peptide (TKRP), neuropeptide-F (NPF), short neuropeptide-F (sNPF), diuretic hormone 31 (DH31), Tenmo-diuretic hormone 37 (Temno-DH37), Allatostatins A (AS-A), Allatostatins B (AS-B, also called myoinhibitory peptides [MIP]), Allatostatins C (AS-C), Allatotropins (AT), and insulin-like peptide 3 (Ilp3) are present in enteric endocrine cells in varying

regions of the midgut in several insect species, understanding their respective roles in digestion is still under investigation (Brown et al., 1986; Brugge et al., 2009; Hernandez-Martinez et al., 2005; Moffett and Moffett, 2005; Onken et al., 2004; Predel et al., 2010; Reiher et al., 2011; Stanek et al., 2002; Veenstra et al., 2009; Veenstra et al., 2008; Veenstra et al., 1995; Veenstra et al., 1997a; Wiehart et al., 2002; Winther and Nassel, 2001).

After being transported or diffusing across the midgut epithelium, the absorbable components of the blood meal (water, ions, and small molecules) are removed from the hemolymph by the mosquito's five Malpighian tubules, the insect renal organs. The Malpighian tubules are distal blind-ended tubes composed of a one-cell thick epithelium containing two cell types: principal and stellate cells (Mathew and Rai, 1976). Principal cells, abundant along the entire length of the tubule, are large, cuboideal cells with prominent nuclei and a thick brush border containing copious mitochondria. Stellate cells are less abundant, smaller and star-shaped, and are located in the distal two thirds of the tubules (Patrick et al., 2006). Between these two types of cells are selectively permeable septate junctions, ladder-like junctions on the lateral cell membranes that are similar to vertebrate tight junctions (Beyenbach, 2003b; Beyenbach et al., 2010). The adult Malpighian tubules are not innervated, but rather, are under hormonal control. After stimulation by diuretic hormones, ions (K^+, Na^+, and Cl^-) are transported across the epithelium into the tubule lumen (for a recent model of diuretic hormone modulated ion transport see Lu et al., 2011). Water follows this osmotic gradient, producing primary urine, which is nearly isoosmotic to the mosquito's hemolymph (Beyenbach et al.,

2010). Primary urine formation from ion and fluid flux into the lumen drives further fluid movement through the excretion system and out of the body.

Key to excretion of excess fluid and ions is selective absorption and reabsorption. Absorption of fluid and ions from the hemolymph occurs along the distal end of the Malpighian tubules. Near the proximal end, void of stellate cells and nearing the pylorus, reabsorption begins (Beyenbach, 1995). Reabsorption continues as the fluid and ions travel through the hindgut, comprised of the ileum and rectum, before it is excreted from the body. Differing from the thin epithelium of the Malpighian tubules, the wall of the rectum has three layers: a thick surrounding muscular layer with circular and longitudinal fibers, rectal epithelial cells, and an inner, apical cuticle. The muscular layer functions in applying a peristaltic contraction to the hindgut to move urine through the tissue and out of the body. Notable in the rectum are pear-shaped ultrastructures called rectal papillae (Figure 2).

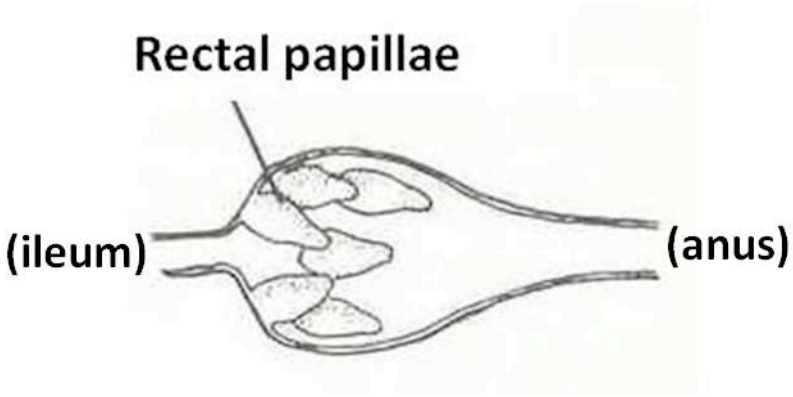


Figure 2. The mosquito rectum. The female mosquito rectum contains six rectal papillae and is involved in ion and fluid reabsorption and excretion (adapted from Clements, 1992).

The female's six rectal papillae have highly folded basolateral membranes, providing significant surface area for reabsorption (Bradley, 1987; Hopkins, 1967). The rest of the rectal sac epithelium is made of simple, squamous cells. To date, while several studies have investigated the general morphology of the adult *Ae. aegypti's* rectum, only two studies have investigated specific ion transport in this organ (Patrick et al., 2006; Kang'ethe et al., 2007). These studies localized the P-typeNa^+/K^+-ATPase and V-type H^+-ATPase to the papillae's basal and apical membranes, respectively, and the ion exchanger NHE3. Thus, rectal physiology at the molecular level has been largely unexplored in adult mosquitoes.

1.5 Neuropeptides and the leucokinin-like family of signaling molecules

In mosquitoes, the activities of these anatomically and functionally coordinated organs, the midgut, hindgut, and M. tubules, are precisely orchestrated by hormones. Given the importance of proper postprandial diuresis to overcome fitness costs associated with the large blood meal, it is necessary to understand which hormones are critical to the process and their respective roles in diuresis. While several diuretic hormones have been identified in the mosquito, leucokinin-like neuropeptides are of particular interest because of their multifunctional myotropic and diuretic activities (Nachman et al., 2009).

Leucokinins were originally isolated from the cockroach, *Leucophaea maderae,* brain and discovered to stimulate hindgut contractions (Holman et al., 1986a; Holman et

al., 1986b; Holman et al., 1987a; Holman et al., 1987b). They were later demonstrated to also induce diuresis in isolated M. tubules of *Aedes aegypti* by affecting ion flux across the epithelium, specifically increasing chloride conductance from the hemolymph toward the M. tubule lumen (Hayes et al., 1989; Pannabecker et al., 1993). In 1994, a leucokinin-like peptide was isolated from a hematophagous insect for the first time (Hayes et al., 1994). That same year, three leucokinin-like peptides were isolated and identified in *Ae. aegypti* as Asn-Ser-Lys-Tyr-Val-Ser-Lys-Gln-Lys-Phe-Tyr-Ser-Trp-Gly-amide, Asn-Pro-Phe-His-Ala-Trp-Gly-amide, and Asn-Asn-Pro-Asn-Val-Phe-Tyr-Pro-Trp-Gly-amide, *Aedes* kinins I, II, and III respectively (Veenstra, 1994). Interestingly, a single cDNA encodes all three of these peptides, which interact independently with a single kinin receptor and can stimulate both hindgut contractions and fluid and ion flux through the Malpighian tubules (Cady and Hagedorn, 1999b; Lu et al., 2011; Pietrantonio et al., 2005; Schepel et al., 2010; Veenstra et al., 1997b). To date, leucokinin-like peptides have been identified in numerous arthropods. As they have been conserved across such a diverse array of taxa, they likely play an integral role in homeostasis, making them critical for survival.

1.6 G protein-coupled receptors and the leucokinin receptor

The *Aedes* kinins signal through the leucokinin receptor (*Ae*KR), a multiligand receptor that has been cloned and sequenced (Protein ID AAT95982.1) from female *Ae. aegypti* Malpighian tubules (Pietrantonio et al., 2005). The predicted receptor is a 584 amino acid residue (65.2 kDa) transmembrane protein that signals through increasing intracellular inositol triphosphate (IP_3). The elevation of cytoplasmic IP_3 concentration then increases intracellular calcium levels by the release of calcium from IP_3-sensitive intracellular calcium stores (Figure 3) (Cady and Hagedorn, 1999a; Pietrantonio et al., 2005). The increased intracellular calcium concentration leads to chloride ion conductance across the membrane from the hemolymph into the tubule lumen. Electrophysiological experiments have shown that simultaneously to the chloride ion movement into the tubule, the transepithelial voltage and electrical resistance across the membrane drop significantly after kinin peptide application to the tubules (Beyenbach et al., 2010). To account for these nearly short-circuited voltage and resistance readings occurring with switch-like speed, it has been hypothesized that kinin stimulated chloride conductance occurs through transient opening of septate junctions between the stellate and principal cells to permit paracellular movement of the ions (Beyenbach, 2003b; Beyenbach et al., 2009). However, this issue is not yet clarified as other findings support the movement of chloride via a transcellular pathway through the stellate cells via the known chloride ion channel on their apical membrane (Figure 3) (O'Donnell et al., 1998; O'Connor and Beyenbach, 2001).

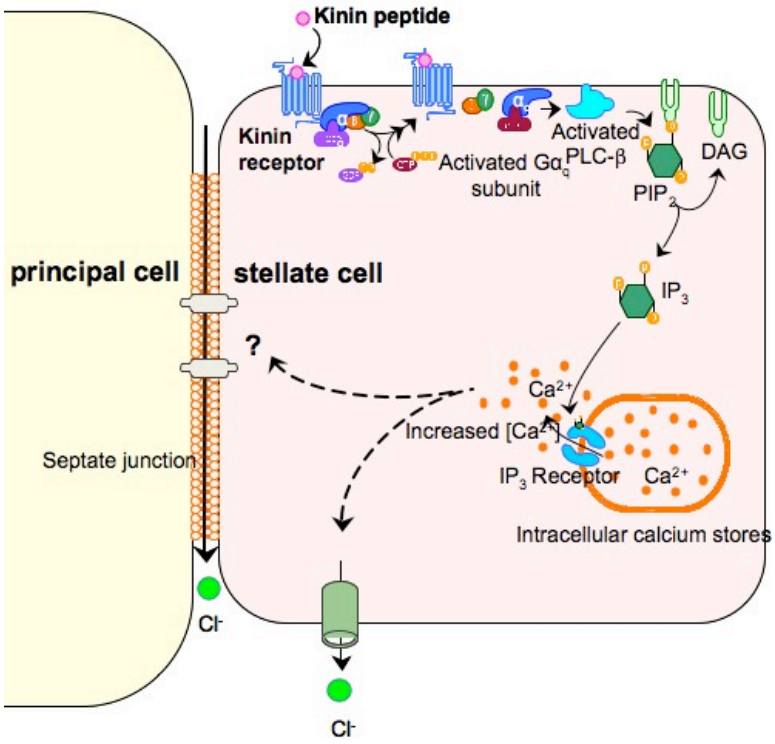


Figure 3. Pathway for kinin activity in *Aedes aegypti*. *Aedes* kinins signaling results in release of intracellular calcium stores (Pietrantonio et al., 2005). *Aedes* kinins bind their respective G protein-coupled receptor (GPCR), the *Aedes* kinin receptor, which activates the $G\alpha_q$ subunit (Holmes et al., 2003). In turn, this activates phospholipase Cβ (PLC-β), which then triggers an increase in intracellular inositol triphosphate (IP_3). The IP_3 binds the IP_3 receptor on the membrane of intracellular calcium stores, releasing calcium into the cytoplasm. Increased intracellular calcium concentration leads to chloride ion flux from the hemolymph into the tubule lumen either paracellularly through the transient opening of septate junctions to transcellularly through chloride channels in the stellate cell membrane.

The *Ae*KR belongs to the family of G protein-coupled receptors (GPCRs). GPCRs are cell membrane signaling proteins, characterized by seven hydrophobic transmembrane domains, an extracellular N-terminus that is usually glycosylated, and a

typically phosphorylated intracellular C-terminus (Brody and Cravchik, 2000; Kobilka, 2007). These receptors, when bound by their respective ligands (a diverse array of molecules including hormones, peptides, proteins, neurotransmitters and ions), undergo a conformational change. This change modulates transmission of a message across the cell membrane through a heterotrimeric guanine nucleotide-binding regulatory protein (G protein). The signal reaches the appropriate intracellular destination via a cascade of secondary messengers.

GPCRs help control critical insect functions such as homeostasis, development, water balance, muscle activity, and behavior (Scherkenbeck and Zdobinsky, 2009), all that relate to adaptations of blood feeding. Given their involvement in modulating aspects of nearly all physiological processes, GPCRs are very attractive potential chemical targets. Furthermore, disrupting the receptor-ligand interaction is hypothetically a very straightforward and practical approach for engineering insecticides. Already, many human GPCRs are targets for modern medicines. These characteristics make the *Ae*KR an exciting and promising protein to study.

Work to understand the contribution of the *Ae*KR to physiological processes in *Ae. aegypti* has focused mainly in the Malpighian tubules. As the receptor is known to function in chloride transport across the Malpighian tubule epithelium, elucidating its specific location has been a widely studied and debated issue. Initial electrophysiology studies in *Ae. aegypti* suggested the *Ae*KR's location on principal cells in the Malpighian tubules, however recent work by Lu et al., (2011) immunolocalized the receptor to the stellate cell basal membrane, similar to reports in both *D. melanogaster* and *A. gambiae*

(Beyenbach, 2003a; Beyenbach et al., 2009; Lu et al., 2011; Radford et al., 2002; Radford et al., 2004; Yu and Beyenbach, 2002; Yu and Beyenbach, 2004). While leucokinin-like peptides are also known to have myotropic effects on the hindgut, little work has been done to study the *Ae*KR in the hindgut.

In addition to their involvement in physiological functions in the Malpighian tubules and hindgut, leucokinins and leucokinin-like peptides have also been found in other tissues including the midgut, ovaries, testes, and nervous system (including the brain and neurosecretory cells of the abdominal ganglia) in several insect species, such as *Anopheles gambiae*, *Leucophaea maderae*, *Periplaneta americana*, *Locusta migratoria*, *Acheta domesticus*, *Culex salinarius*, *Helicoverpa zea*, *Musca domestica*, *Drosophila melanogaster*, and the tick *Boophilus microplus* (Blackburn et al., 1995; Cantera and Nässel, 1992; Clottens et al., 1993; Coast et al., 2002; Cook et al., 1989; Harshini et al., 2002; Hayes et al., 1994; Holman et al., 1984; Holman et al., 1999; Holman et al., 1991; Holman et al., 1990; Holmes et al., 2003; Predel et al., 1997; Radford et al., 2002; Radford et al., 2004; Schoofs et al., 1992; Terhzaz et al., 1999; Veenstra, 2009). A review has compared the known kinin peptides (Torfs et al., 1999). The known leucokinin receptors from six species were aligned for identity and similarity in 2004 (Radford et al., 2004). Unpublished data from Dr. Pietrantonio's laboratory has also found the *Ae*KR transcript to be highly abundant in the ovary. To my knowledge, the *Ae*KR has otherwise never been found or studied in any mosquito reproductive organ.

1.7 Integrative signaling molecules

As the *Ae*KR has been identified in both the excretory and reproductive systems of *Ae. aegypti* and in the digestive and nervous systems in several other arthropod species, the *Ae*KR may play an integrative role in endocrine signaling of physiological changes that occur post blood feeding, and thus exhibit pleiotropy (Figure 4). This possibility makes further assessment of the receptor's location and function an appealing direction for future research.

The focus of this Master of Science research is to understand the distribution the *Ae*KR in the key digestive, excretory, and reproductive organs and to begin elucidating its role in mosquito fluid excretion post blood feeding. Further, while there is a plethora of techniques available to study transcriptomics, mRNA presence is still one step removed from the functional protein, and thus the associated phenotypes. While proteomics methods are highly limited and strenuous, compared to transcriptomics, the data has the capacity to provide more direct analysis of gene function and expression. Thus, studying a protein, specifically for this study the *Aedes* kinin receptor, in these mosquito systems will provide significant insight into the receptor's possible integrative role.

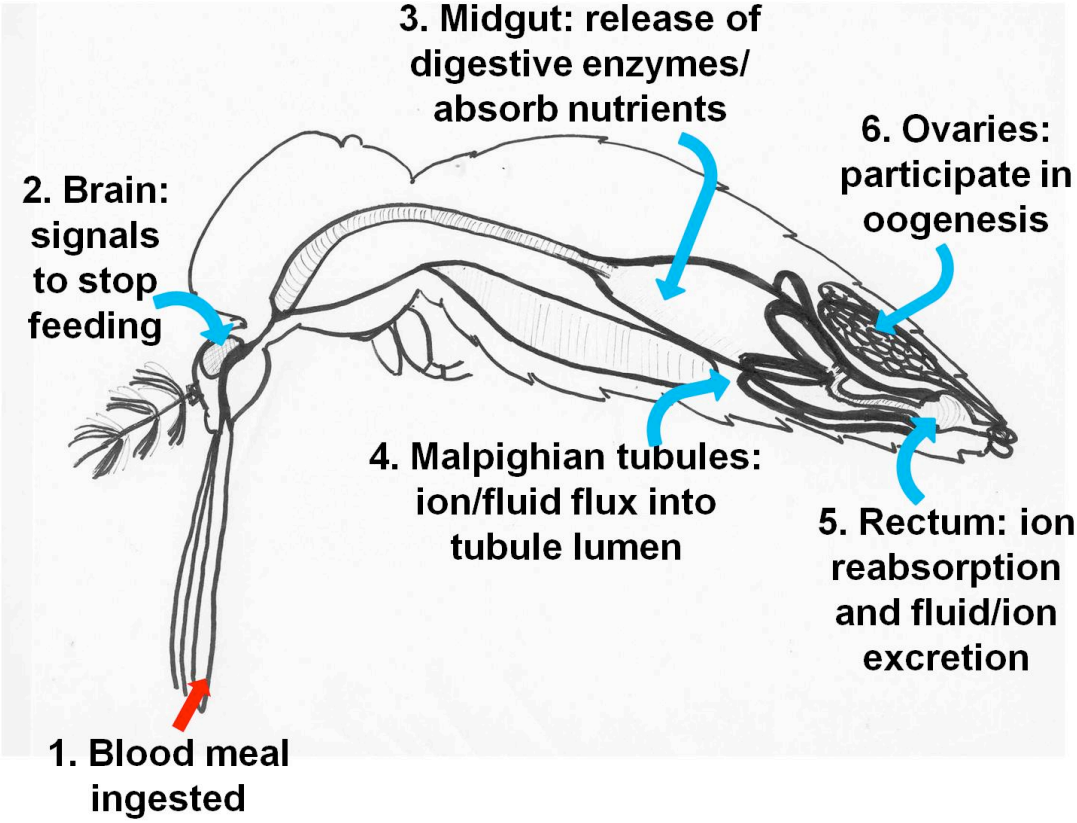


Figure 4. Female physiological changes after a full blood meal (adapted from Clements, 1992). The evolution of the blood feeding adaptation in mosquitoes resulted in the coordination of multiple physiological processes such as host seeking behavior, diuresis, digestion, and oogenesis. While proteins from the blood meal are essential for reproduction, females ingest more than ten times their hemolymph volume during feeding, hindering flight and threatening hemolymph homeostasis. To overcome these potential fitness costs females rapidly excrete excess ions and fluid while retaining nutrients for oogenesis. As these processes are under synchronous hormonal control, it is possible the same peptides regulate multiple organs.

This study employs protein studies to answer the following question. *Where is the kinin receptor located in tissues important to blood feeding processes and what is its function in diuresis?*

Hypothesis: The kinin receptor will be found in the key digestive, excretory and reproductive tissues and head. It functions to increase fluid excretion rates during the post-peak phase of rapid diuresis.

This hypothesis was tested by investigating the following three objectives:

1) Setting up a reliable, blood feeding system capable of feeding variable numbers of mosquitoes (one to several hundred females) for experiments on single insects and colony maintenance.
2) Conducting a global expression analysis of the *Ae*KR protein in the female mosquito *Ae. aegypti*. The analysis focused on the Malpighian tubules, midgut, hindgut, head, and ovary, and was completed by western blot and immunohistochemistry.
3) Knocking-down *Ae*KR expression by RNA interference (RNAi) and observing phenotypic changes in fluid excretion rate and volume. Knockdown success levels were determined by quantitative RT-PCR (qPCR) and immunohistochemistry; *Ae*KR influence on fluid excretion rates and overall volume excreted was measured in a precision humidity chamber.

To my knowledge this is the first study to identify the *Aedes* kinin receptor in the midgut, hindgut, ovaries, and head of female *Ae. aegypti*, as well as the first study to show the receptor's *in vivo* contribution to post-prandial diuresis.

CHAPTER II

OBJECTIVE 1:

SET UP AN ARTIFICIAL BLOOD FEEDING SYSTEM

2.1 Introduction

Blood feeding is central to studying mosquito biology. As blood feeding is key for the physiological processes being studied in this thesis, many of the experiments require the availability of a reliable blood feeding system. Until now, mosquito colonies have been maintained by feeding adult females on chickens or mice. However, it is exceedingly difficult, if not impossible, to feed a single mosquito on a chicken and then immediately begin recording diuresis. While this is somewhat possible using mice, mosquitoes can take a relatively long time to initiate feeding so too many mice would be needed. This is inconvenient, but it is dictated by government regulations on the length and frequency of anesthetizing the mice. Thus, an artificial feeding system has been set up in the laboratory to expedite the RNAi and fluid excretion experiments.

Blood feeding systems utilizing water-jacketed feeders have been successfully established in several laboratories. Originally designed and described in Rutledge et al. (1964), the feeders can be useful for both colony maintenance and single insect feeding.

Hypothesis: Females will feed on defibrinated blood until fully engorged using artificial feeders and then oviposit viable eggs.

2.2 Materials and methods

Custom glass jacketed membrane feeders (Chemglass Life Sciences, NJ, USA), were set up in a circuit with lab-grade, clear, plastic PVC tubing (3/32" x 1/32") (Figures 5,6). Feeders were connected to a variable flow rate pump (VWR, Radnor, PA, USA) and to a glass beaker filled with water kept at approximately 50^{o}C by a hot plate. At this temperature water cooled to the appropriate temperature (~37^{o}C) by the time it reached the feeders. Defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA) or fresh bovine blood (Rosenthal Meat Science Center, Texas A&M University, College Station, TX, USA) defibrinated by gentle agitation (Appendix A), was supplemented with 1.67 μg ATP (Sigma-Aldrich, St. Louis, MA, USA) per 1 ml blood. Parafilm membranes were stretched over the bottom opening on the feeders, and then thinly coated with Vaseline (Vaseline, NY, USA) (Figure 7). From 100 μl to 400 μl blood was applied to each feeder, depending on the number of mosquitoes to be fed. Feeders were placed directly on top of the cages containing female *Ae. aegypti*.

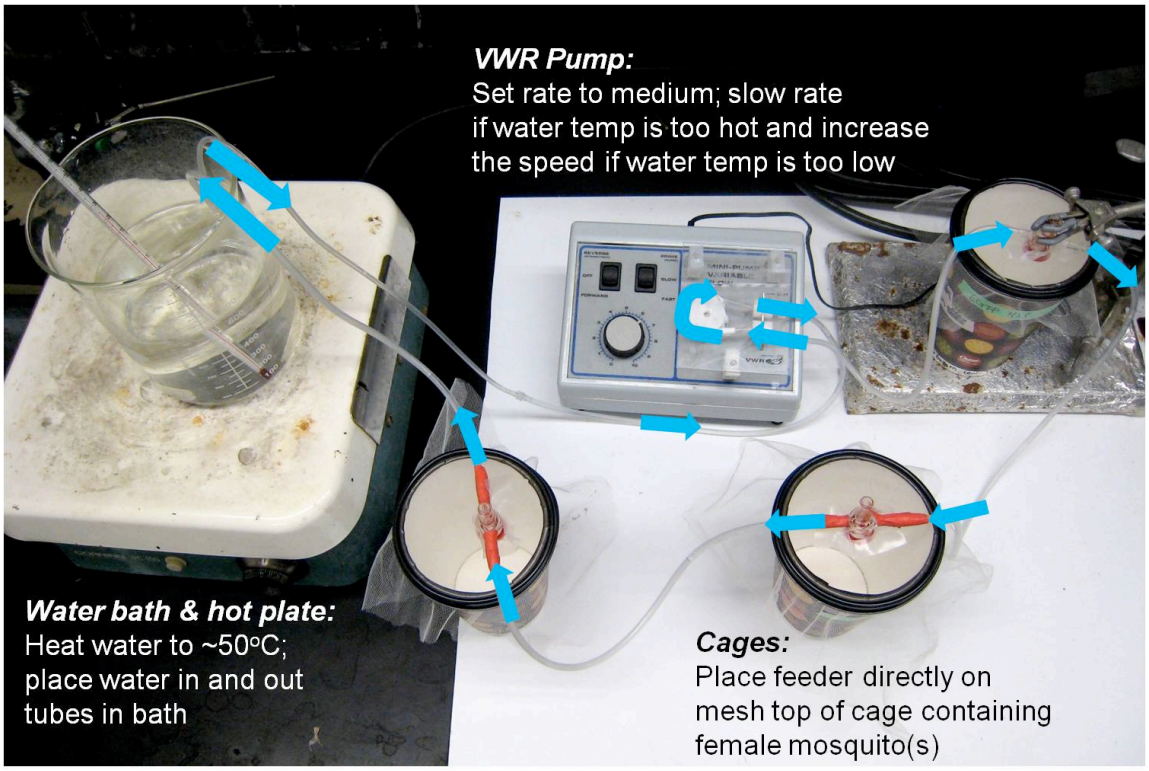


Figure 5. Water flow through the artificial blood feeding system set-up. Water in a 1000 ml beaker was heated to approximately 50°C on a hotplate. A variable rate pump drove hot water to leaves the water bath (beaker) through PVC tubing, which then circulated through the pump apparatus and into the first feeder. Water in the first feeder warmed the blood to around 37°C (human body temperature). Water left the first feeder and repeated the heating process for blood in two subsequent feeders, attached in series. Water, then cooled to close to room temperature, was recycled back into the water bath where it was heated to 50°C and the cycle repeated. Blue arrows indicate direction of water flow.

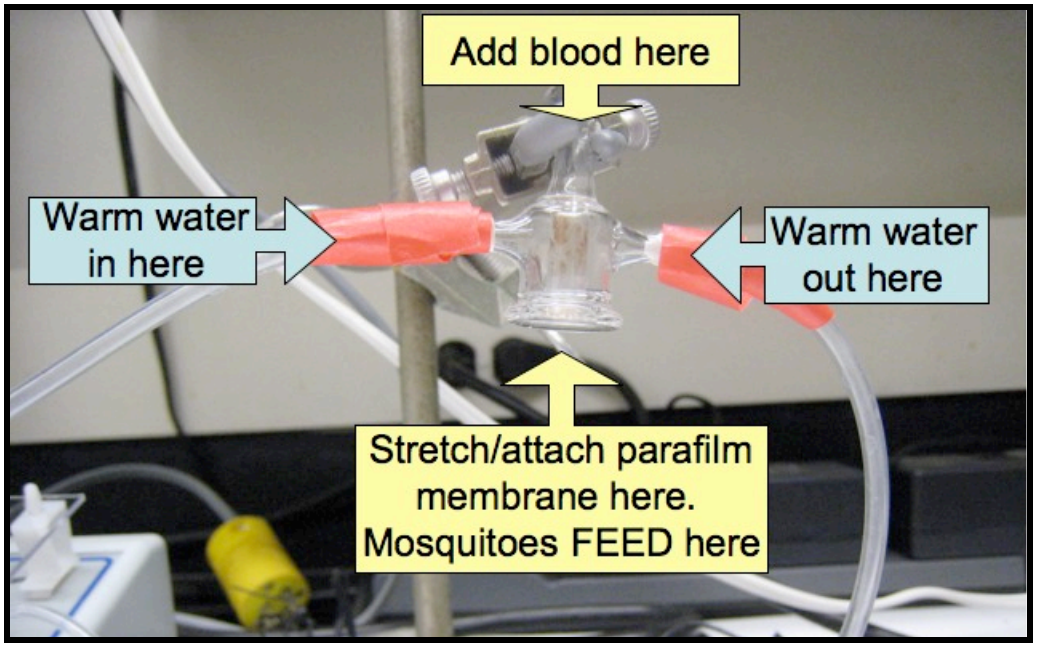


Figure 6. Glass water-jacketed membrane feeder. After a parafilm membrane was applied to the bottom, blood was added through the top hole. Warm water entered from the left arm, circulated through the outer chamber to warm the blood located in the inner chamber, and then exited through the right arm.

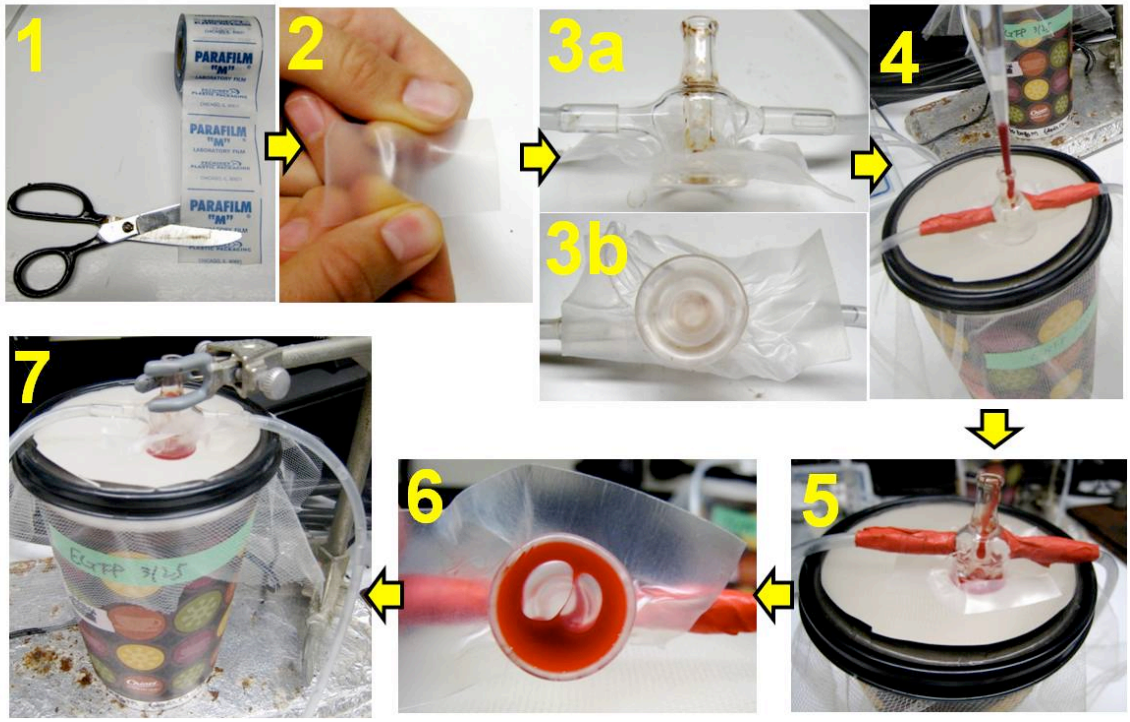


Figure 7. Artificial membrane blood feeder preparation. (1) Parafilm was cut into approximately 2 cm wide strips. (2) Cut parafilm was stretched along its short axis as pictured. (3) The stretched parafilm was applied to the bottom of the blood feeders and adhered to the glass to ensure no blood leakage. A thin layer of Vaseline was coated onto the parafilm after it was stretched over the bottom of the feeder. (4,5) The feeder was placed on the mesh top of a cage containing the female mosquitoes to be fed and blood was pipetted (~100-600 µL) into the feeder through a thin tube opening on the top. (6) The blood was visualized from the bottom to ensure there was a working seal around the rim and there were no holes in the membrane. (7) The feeder, equipped with blood was placed on top of a cage, which allowed female(s) access to the meal.

2.3 Results and discussion

Using the above described artificial feeding system, female mosquitoes were successfully fed to completion in both isolation (1 mosquito per cage) and in crowded conditions (greater than 100 mosquitoes per cage) showing that that number of mosquitoes present did not affect their ability or propensity to feed on the membranes. The mosquitoes fed on both purchased defibrinated rabbit blood (Hemostat Laboratories) and on freshly defibrinated bovine blood. Defibrinated blood is blood without fibrin, a protein involved in the process of blood clotting. Blood without fibrin was stored at 4^{o}C without clotting for later use. There are alternate methods to keep blood from clotting, such as adding anticoagulants to fresh blood samples, however because this study aims to better understand physiological processes in the mosquito, anticoagulants could not be used because they could possibility alter normal mosquito physiology. Thus, defibrination was the best option as it provided a blood meal for female mosquitoes similar to natural vertebrate blood.

Two sources of blood were evaluated in this study. First, commercial defibrinated rabbit blood was tested. Defibrinated rabbit blood (30 ml; DRB030 was shipped overnight from HemoStat laboratories). This was an excellent source when blood was needed quickly and it could not be collected on the TAMU campus. Alternately, bovine blood could be obtained from the Rosenthal Center (Meat Sciences, Texas A&M University, College Station, TX) and defibrinated in the laboratory (Appendix A). Collecting bovine blood could only be done during pre-scheduled

slaughters determined by the Meat Sciences Department (TAMU), which limited this method's reliability and consistency. A simple protocol was prepared that provides a method to obtain at least 500 ml of defibrinated bovine blood for research purposes (Appendix A). Briefly, blood was collected during a beef slaughter at Rosenthal Meat Science Center (TAMU) and then was defibrinated in the laboratory by gentle agitation with sterile glass marbles. Liquid blood lacking fibrin was then pipetted from around a large blood clot into separate, sterile containers and stored in 50 ml aliquots at 4^{o}C. Both of these sources worked well for feeding as female *Ae. aegypti* fed readily on bovine blood for approximately three weeks after it is was collected and on commercially purchased defibrinated rabbit blood for approximately 3 weeks after ordering. Beyond these three weeks mosquitoes would not feed on either blood source.

Immediately prior to feeding, ATP was added to defibrinated blood (both rabbit and bovine) (Rutledge et al., 1964). The concentration of ATP required for feeding was variable (~1–2 μg ATP per 1 ml blood was successful), but females would not feed without additional ATP, as expected based on previous reports. The nutrient state of females prior to feeding appeared to have little effect on the success of the blood feeding. For instance, females blood-fed after starvation for 12-24 hours, after being fed with cotton feeders saturated with a 10% dextrose solution, and with these sugar-water feeders still present in the cage. After being starved for more than 24 hours the females would not feed, at which time they appeared too weak to feed. After feeding (on either rabbit or bovine blood) females reared to oviposition were capable of ovipositing and the larvae were viable and able to reproduce.

In summary, protocols were established for both bovine blood defibrination and membrane feeder set-up and use (Appendices A and B). This artificial blood feeding system was able to efficiently feed female *Ae. aegypti* as hypothesized. This system was readily applied to objectives in further chapters and it will be highly useful for future studies and colony maintenance in the laboratory.

CHAPTER III

OBJECTIVE 2:

GLOBAL TISSUE ANALYSIS OF THE *AEDES* KININ RECEPTOR

3.1 Introduction

Evolution of the blood feeding adaptation has required precisely coordinating multiple physiological systems including behavior, digestion, excretion, and oogenesis (Klowden, 1995; Waage, 1979). Protein from the blood meal is critical for reproduction. However, during blood feeding, mosquitoes ingest more than ten times their hemolymph volume, imposing a fitness cost as the added weight hinders flight and the differing ion composition and hypoosmotic character of the blood threatens hemolymph homeostasis. Thus, females initiate rapid diuresis while essential nutrients are retained for oogenesis. These physiological processes occur concurrently and are under precise, synchronous hormonal control (Adams, 1999). It is therefore plausible that hormones known to modulate functions such as diuresis, and are therefore distributed throughout the mosquito's open circulatory system, may have undiscovered simultaneous actions on other organs, rendering them integrative signaling molecules.

While numerous hormones function after a blood meal, leucokinin-like neuropeptides are of particular interest because of their multifunctional myotropic and diuretic activities and their potential role in digestive enzyme release (Nachman et al., 2009). In *Ae. aegypti* three leucokinin-like peptides have been identified, *Aedes* kinins I,

II, and III, which interact independently with a single *Aedes* kinin receptor (*Ae*KR) and can stimulate both hindgut contractions and fluid and ion flux through the Malpighian tubules for primary urine production (Cady and Hagedorn, 1999b; Pietrantonio et al., 2005; Schepel et al., 2010; Veenstra, 1994; Veenstra et al., 1997b). The *Ae*KR (Protein ID AAT95982.1) is predicted as a 584 amino acid residue (65.2 kDa) G protein-coupled receptor (GPCR) that has been shown to signal through intracellular Ca^{2+} (Cady and Hagedorn, 1999a; Pietrantonio et al., 2005). To date, work to understand the contribution of the *Ae*KR to physiological processes in *Ae. aegypti* has focused on the Malpighian tubules, where it was recently immunolocalized to stellate cells (Beyenbach, 2003a; Beyenbach et al., 2009; Lu et al., 2011; Radford et al., 2002; Radford et al., 2004; Yu and Beyenbach, 2002; Yu and Beyenbach, 2004). In other arthropods, leucokinin-like peptides and/or their receptor have also been discovered in the ovaries, testes, midgut and nervous system (Blackburn et al., 1995; Cantera and Nässel, 1992; Clottens et al., 1993; Coast et al., 2002; Cook et al., 1989; Harshini et al., 2002; Holman et al., 1984; Holman et al., 1999; Holman et al., 1991; Holman et al., 1990; Holmes et al., 2003; Hayes et al., 1994; Predel et al., 1997; Radford et al., 2002; Radford et al., 2004; Schoofs et al., 1992; Terhzaz et al., 1999; Veenstra, 2009). As leucokinin-like peptides and their receptor have been discovered in several key physiological systems and appear to be conserved across a diverse array of taxa, they may play an integral role in homeostasis by integrating endocrine signaling throughout the body, exhibiting pleiotropic activity (Hayes et al., 1989).

This objective aimed to further assessment of the *Ae*KR's location and function in adult, female *Ae. aegypti*. Specifically, the objective sought to assess the receptor's presence and distribution among key digestive, excretory, and reproductive organs: the head, posterior midgut (stomach), hindgut, and ovaries by western blot and immunohistochemistry. To my knowledge, the *Ae*KR has not been studied in the brain, ovaries, or midgut of *Ae. aegypti,* and in the hindgut has not been localized to a specific site.

Hypothesis: The *Ae*KR will be detected in the western blots from all of the tissues selected: head, midgut, M. tubules, hindgut, and ovaries, based on their presence in these organs in other arthropod species. Further, the receptor will be immunolocalized in a plasma membrane in contact with the hemolymph in these tissues because this location would position the receptor to contact and be bound by *Aedes* kinin peptides that are released into the circulating hemolymph after blood feeding.

3.2 Materials and methods

Colony maintenance and dissections. *Ae. aegypti* (L.) Rockefeller strain (Diptera: Culicidae) were reared and maintained as previously described (Lu et al., 2011). Adults were maintained on a 10% dextrose solution *ad libitum*. Females were blood fed using water-jacketed feeders (Chemglass Life Sciences) (Rutledge et al., 1964) with defibrinated rabbit blood (Hemostat Laboratories). M. tubules, head, posterior midgut

(stomach), ovaries, and hindgut were dissected in phosphate buffered saline (PBS) solution under a dissecting microscope. The age and nutrition of the mosquitoes dissected was specified for each experiment.

Antibodies. Polyclonal anti-peptide antibodies against the kinin receptor were previously developed in New Zealand female rabbits (Bethyl Laboratories, Inc., Mongomery, TX, USA), and tested in Dr. Pietrantonio's laboratory (Lu et al., 2011). The rabbit anti-KR-$Ct_{328-345}$ (NEKFKREFHKRYPFRGRN) antibody, targeting a region near the C-terminus after the seventh predicted transmembrane region (Appendix C), was used for all experiments.

Western blot. Membrane preparations and western blots were completed as previously described (Lu et al., 2011). Homogenates of membrane-bound proteins were prepared from the head, stomach, M. tubules, hindgut, and ovaries of non-blood fed (NBF) and blood fed (BF) females at 3-5 d post eclosion (PE) (Table 1). BF dissections were completed 1-3 h post feeding for all indicated tissues except the stomach. Ovaries were also dissected 24 h after feeding. Briefly, tissues were homogenized with a handheld, battery-powered motor homogenizer in cold Buffer A (25mM Tris/HCl, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol (DTT), dH2O) containing Complete Protease Inhibitor Cocktail (1 tablet per 50 mL Buffer A solution; ROCHE) and then centrifuged at 800 g for 5 min at 4^{o}C. The supernatants were collected and then the pellets were resuspended in cold Buffer A, homogenized, and subject to the same

centrifugation. After three to five replicates of homogenization, centrifugation and then collection of the supernatant, the collected supernatants were centrifuged at 100,000 g for 1 h at 4^{o}C. The pellets were resuspended in cold Buffer B (50mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$) with protease inhibitors. Sample concentrations were determined by spectrometry using albumin standards. The membrane preparations were stored at -80^{o}C.

Table 1. Tissues dissected for western blots. post eclosion (PE); non blood-fed (NBF); post blood meal (PBM).

Sample	Tissues	Mosquito age	Nutritional status	Number of females dissected
1	Head	3-5 days PE	NBF	1000
2	Midgut	3-5 days PE	NBF	1000
3	Malpighian tubules	3-5 days PE	NBF	1000
4	Hindgut/rectum	3-5 days PE	NBF	1000
5	Ovaries	3-5 days PE	NBF	1000
6	Head	3-5 days PE	1-3 hrs PBM	1000
7	Malpighian tubules	3-5 days PE	1-3 hrs PBM	1000
8	Hindgut/rectum	3-5 days PE	1-3 hrs PBM	1000
9	Ovaries	3-5 days PE	1-3 hrs PBM	1000
10	Ovaries	3-5 days PE	24 hrs PBM	500

Briefly, the membrane preparations were boiled with loading dye including dithiothreitol (DTT), electrophoresed on SDS-PAGE gels (10% Tris-HCl, Bio-Rad, Hercules, CA, USA) and then transferred to poly-vinylidene difuoride (PVD) membranes (Millipore, Billerica, MA, USA). For NBF tissues 100 µg membranes were

loaded per lane and 50 μg were used for preparations from BF females. After blocking in TBST (10 mM Tris base, 140 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% non-fat milk for 1 h at RT, membranes were be incubated overnight at room temperature (RT) with either rabbit anti-KR-$Ct_{328–345}$ antibodies (1:250) in the blocking solution or rabbit anti-KR-$Ct_{328–345}$ antibodies (1:250 in blocking solution) pre-absorbed with 500 μg C-terminal peptide antigen, as a negative control. PVD membranes were then washed in for 3 x 10 min in TBST, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) (1:40,000) for 1 h at RT, and then washed again for 3 x 10 min in TBST. The banding patterns of the proteins recognized by the anti-peptide antibody were visualized were visualized on film (Kodak, Rochester, NY, USA) using the Enhanced Chemiluminescence System (Pierce, Rockford, IL, USA).

NBF MT and ovary and BF ovary membrane preparations were tested for N-link glycosylation by treatment with PNGase F (New England BioLabs Inc., Ipswich, MA, USA) per the manufacturer's protocol, and then assessed by western blot. Briefly, membrane preparation (20 μg) was combined with 10X Glycoprotein Denaturing Buffer (1 μL) then brought to a total volume of 10 μL with sterile H_2O. Due to the size of the wells in the SDS-PAGE gels (10% Tris-HCl; Bio-Rad) being 50 μL no more than 20 μg of protein could be used in the deglycosylation treatment as the total volume would then exceed the volume of the well the sampled was loaded into. The reaction was heated at 100^{o}C for 10 min and then combined with a solution containing 10X G7 Reaction Buffer (2 μL), 10% NP40 (2 μL), PNGaseF (2 μL) and sterile H_2O (4 μL) and incubated at 37^{o}C

for 1 h. After incubation the reaction was placed on ice and then analyzed by western blot as described above.

Immunohistochemistry. Whole-mount immunohistochemical staining of the posterior midgut, hindgut, and M. tubules from NBF females 3-5 d PE was completed as previously described (Lu et al., 2011). Immunohistochemical staining of tissue sections was completed for posterior midguts from NBF females 3-5 d PE as previously described (Lu and Pietrantonio, 2011). Table 2 shows a summary of the tissues analyzed by immunohistochemistry.

Table 2. Tissues dissected for immunohistochemical staining.

Sample	Tissues	Mount type	Mosquito age	Nutrition	Insects per trial
1	Posterior midgut (Stomach)	Whole and Sections	3-5 days PE	NBF	20
2	M. tubules	Whole	3-5 days PE	NBF	20
3	Rectum	Whole	3-5 days PE	NBF	20

Briefly, dissected tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS. The whole-mount samples were fixed for 2 h at RT with gentle agitation. After being washed in PBS for 5 x 10 m at RT, 70% ethanol for 3 x 10 m at 4^{o}C, again in PBS for 2 x 5 min at RT, treated 12 µg/ml protease K (Sigma–Aldrich) in PBS for 5 min at RT, and washed with PBSTG (PBST (PBS containing 0.01% Tween) containing 2% Normal Goat Serum (NGS)) for 2 x 5 min at RT, the

tissues were incubated with either the rabbit anti-KR-$Ct_{328-345}$ antibody (1:10) or the rabbit anti-KR-$Ct_{328-345}$ antibody pre-absorbed with 500 μg of the C-terminal peptide antigen (1:10) for 24 h at RT in PBSTG (2% NGS). All remaining steps were carried out at RT. Tissues were then washed in PBSTG for 4 x 20 min, and then incubated with Alexa Fluor 546 goat anti-rabbit IgG (1:200 in PBST containing 2% NGS) (Invitrogen, Carlsbad, CA, USA) overnight. After overnight incubation the samples were washed for 6 x 30 min in PBST.

For posterior midgut sections, the organs were fixed in 4% paraformaldehyde for 3 h at RT then embedded in Paraplast-Xtra wax (Fisher Scientific, Waltham, MA, USA). Wax sections (12 μm) were cut using a rotary microtome and mounted onto Superfrost Plus™ slides (Fisher Scientific) and dried for 2 days at 39°C. Per Lu et al. (2009), tissues were de-waxed by incubating for 2 x 5 min in xylene and then rehydrated in 100%, 95% and 70% ethanol for 10 min each and then in water for 30 min at room temperature. Slides were then rinsed in PBS containing 0.05% Triton X-100 followed by incubation with blocking solution (PBST with 5% goat serum and 0.5% bovine serum) for 1 h at room temperature. Next, the slides were incubated overnight in a 4°C wet chamber with rabbit anti-KR-$Ct_{328-345}$ (1:10 in blocking solution) or negative controls (in blocking solution). Negative controls were either pre-immune serum from the same rabbit (1:40) or rabbit anti-KR-$Ct_{328–345}$ antibodies pre-absorbed with the C-terminal peptide antigen (500 μg per 40 mg antibody) for 5 h at RT.

For both whole mount tissues and tissue sections, after incubation with the primary antibody or control, the tissues were washed in PBSTG for 4 x 20 min,

incubated with biotinylated goat anti-rabbit IgG (1:200 in their respective blocking solutions) (Jackson ImmunoResearch Laboratories) for 90 min, washed in PBSTG for 4 x 20 min, then incubated with Streptavidin-Alexa Fluor 546 (1:200 in their respective blocking solutions) (Invitrogen, Carlsbad, CA, USA) for 1 h, and then the washed for 6 x 30 min in PBST.

Both whole-mount and section samples were mounted with Vectashield^{TM}-DAPI (Vector Laboratories, Burlingame, CA, USA) and observed under a Carl Zeiss Axioimager A1 microscope with filters for DAPI and Alexa Fluor 546, and equipped with an AxioCam MRc color camera and axiovision program for image analysis. Confocal images were taken of whole-mount tissues using an Olympus FV1000 confocal microscope at the Microscopy and Imaging Center (TAMU, College Station, TX, USA).

3.3 Results and discussion

Western blot confirmed the presence of the *Ae*KR in the M. tubules, head, stomach, hindgut, and ovaries of non blood fed (NBF) and blood fed (BF) females (Figure 8). A 75 kDa band, though faint in the NBF and 1-3 h PBF ovaries, was observed in all of the tissues. This band, 10 kDa higher than the predicated protein size of 65 kDa, has been previously observed in the M. tubules where the size difference was attributed to post-translational modifications (Lu et al., 2011; Pietrantonio et al., 2005). Treatment with PNGaseF to test for N-linked glycosylation of the receptor revealed no shift in band size (not shown).

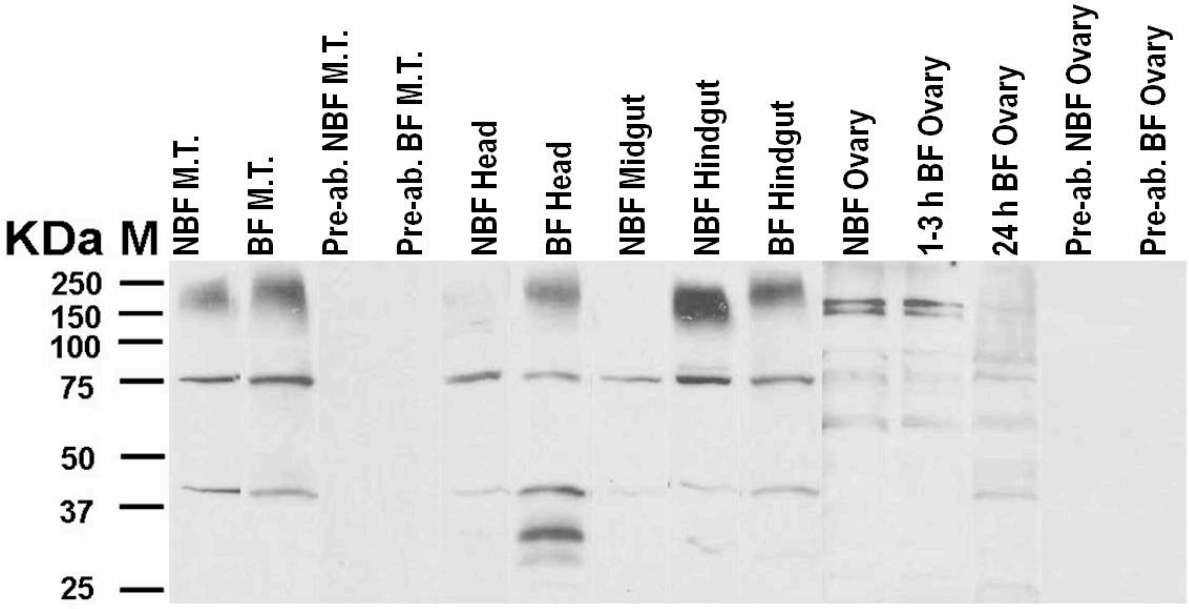


Figure 8. Western blot analysis of the *Ae*KR. The receptor was labeled in membrane preparations from non blood fed (NBF) and blood fed (BF) adult females' M. tubules (MT), heads, stomachs, hindguts, and ovaries with a rabbit anti-KR-$Ct_{328–345}$ antibody, according to standard protocol. Rabbit anti-KR-$Ct_{328–345}$ antibody preabsorbed with the C-terminal peptide antigen (Pre-ab.) served as a negative control. The expected 75 kDa protein band was observed in all samples labeled with the rabbit anti-KR-$Ct_{328–345}$ antibody. Additional bands are observed in the ovary perhaps suggesting tissue specific receptor post-translational modifications or receptor aggregation, dimerization, or binding to other unknown proteins.

As GPCRs are well known to undergo numerous types of post-translational modifications, other modifications may explain the observed disparity between the predicted and observed bands (Brody and Cravchik, 2000; Gether, 2000; Kobilka, 2007). Pietrantonio et al. (2005) noted three potential ASNglycosylation sites using the PPSEARCH' program, and using the NetPhos 2.0 server identified 34 prospective phosphorylation sites and 5 possible palmitoylation sites in the *Ae*KR's amino acid sequence. Additionally, based on the specific orientation of glycosylated residues and their neighboring amino acids and modifications, enzymatic activity to remove these links may have been inhibited because it is known that PNGase F is most active in certain amino acid sequence contexts and does not cleave bonds near other residue sequences. A weaker band around 40 kDa was also resolved in all samples except the NBF and 1-3 h PBF ovaries, which has previously been labeled a degradation product; the presence of other isoforms of the receptor were ruled out by extensive cloning and sequencing (Lu et al., 2011).

Additional bands were observed in the ovaries (NBF and BF), which raise the possibility of alternate posttranslational modifications. As posttranslational modification of GPCRs is known to regulate their activity (Nikitenko et al., 2006), alternate modification of the *Ae*KR in the ovaries may regulate the receptor's tissue-specific function. Posttranslational modification in the ovary may result in multiple forms to regulate the function and/or activation of the receptor in the ovaries to precisely coordinate the timing and regulation of ovarian development. Posttranslational modification of GPCRs is known to regulate their activation and functions (Nikitenko et

al., 2006). To date, the only leucokinin-like receptor identified in the ovaries was the Drosokinin receptor, which was identified by western blot, but was not immunolocalized or studied to determine its function in this tissue (Radford et al., 2002). Due to apparently low *Ae*KR expression in the ovary as well as background labeling by the secondary antibody we were unable to distinguish a specific *Ae*KR labeling pattern in the ovaries above background florescence in immunolocalization experiments. Thus, the receptor's location and function in the ovary remains unknown for any insect.

These western blot results confirm the presence of the *Ae*KR in all of the tested tissues, supporting previous reports from *Drosophila* where the receptor has been identified in the adult head by western blot and immunohistochemical staining, in the ovaries by western blot, and in the larval midgut by IHC (Radford et al., 2002; Veenstra, 2009). This is the first report identifying the receptor in the head, midgut, ovary, or hindgut of any mosquito species.

Immunohistochemical staining of the *Ae. Aegypti* stomach (posterior midgut) localized the *Ae*KR to cone-shaped enteric endocrine cells in the female posterior midgut (Figure 9). A gradient in the number of immunoreactive cells was notable across the stomach, with a greater number of posterior cells expressing the receptor versus anterior cells. The immunoreactive endocrine cells have a patchy distribution between the epithelium's taller columnar cells (Figure 9c,d), which showed no fluorescence. Suitably, pre-absorbed rabbit anti-KR-$Ct_{328-345}$ antibody controls showed no immunoreactivity (Figure 9b). As multiple peptides have been immunolocalized to specific cells and isolated regions of the midgut's 500 endocrine cells, further co-immunlocalization studies would illuminate the contents of the specific cells expressing the *Ae*KR to clarify the *Aedes* kinins' role in the midgut (Brown et al., 1986; Brown et al., 1985; Hernandez-Martinez et al., 2005; Onken et al., 2004; Moffett and Moffett, 2005; Predel et al., 2010; Stanek et al., 2002; Veenstra et al., 1995; Veenstra et al., 1997a). Previous reports demonstrated that leucokinin-like peptides influence digestive enzyme release in larval *Opisina arenosella* midguts, therefore a similar function may be possible for *Aedes* kinins (Harshini et al., 2002). If so, these peptides would not only be 'myotropic and diuretic hormones' as they are currently classified, but would also modulate blood meal digestion, potentially acting as tropic hormones.

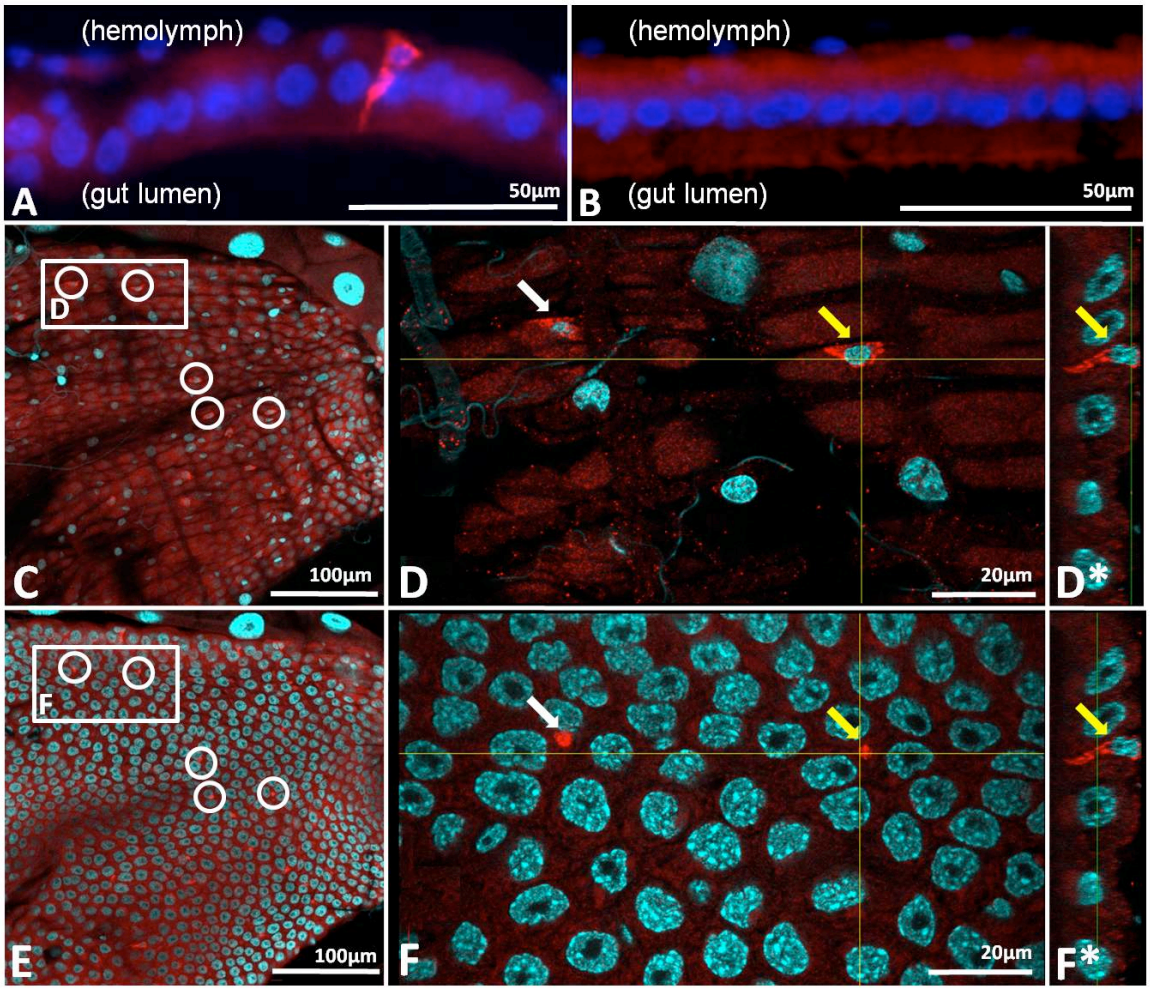
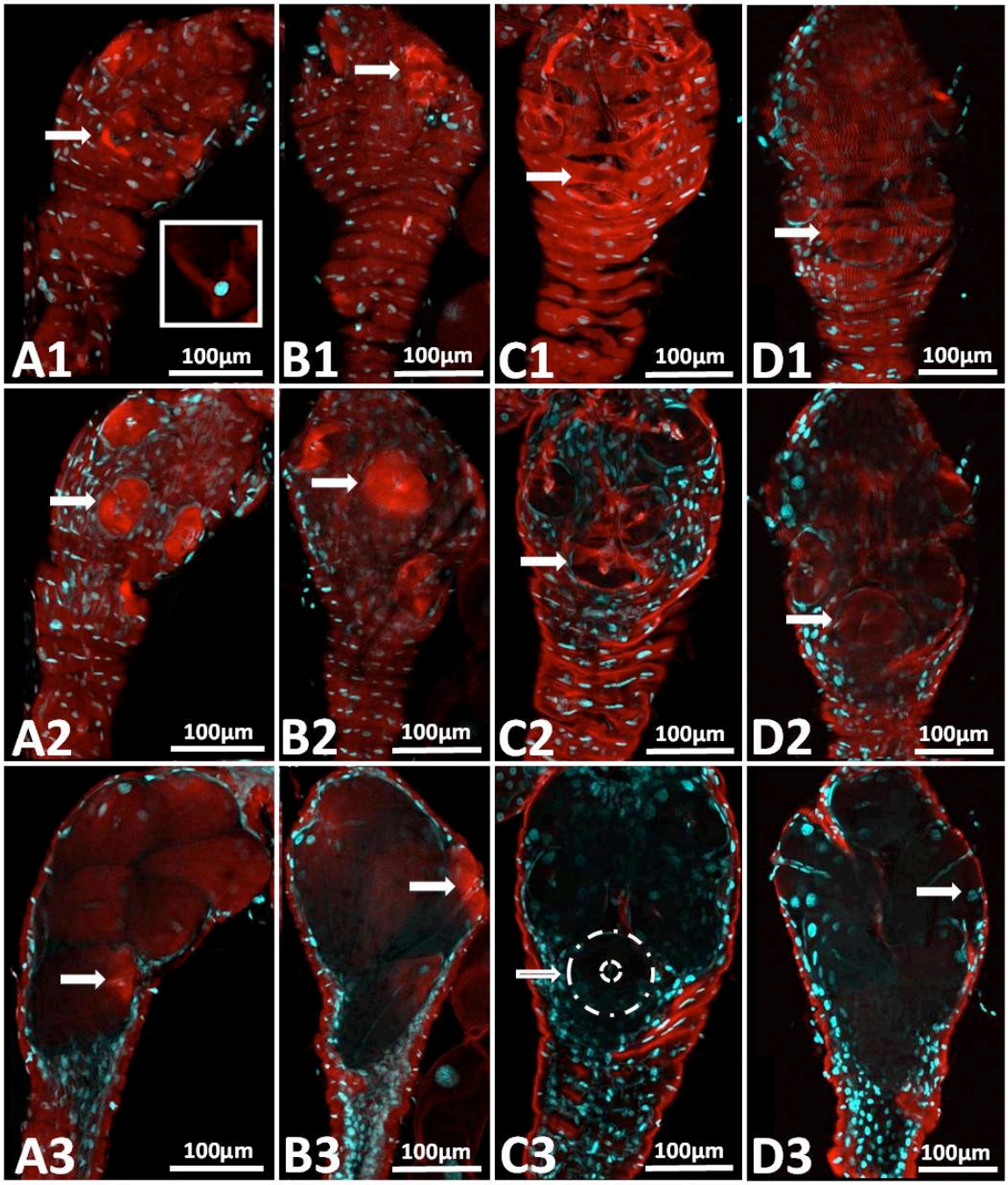


Figure 9. Immunolocalization of the *Ae*KR in the stomach. (A) Receptor signal (red) was observed in cone-shaped endocrine cells in wax-sections under florescence microscopy. (B) Incubation with pre-absorbed antibodies showed no immunoreactivity in endocrine cells. (C-F) Receptor signal in endocrine cells in whole-mounts of stomachs analyzed by confocal microscopy. (C) Immunoreactive endocrine cells display a patchy distibution (hemolymph side). (D) Close-up of endocrine cells, hemolymph side (white inset in C). (D*) Stacked images (47 optical sections, 0.41 µm/section) show basal location of single image (D) in the midgut epithelium; the vertical green line in D* corresponds to vertical yellow cross-hair in D. (E) Single confocal image (1.9 µm/section) through the middle of the epithelium shown in C, red signal indicates the tails of endocrine cells. (F) Close-up of endocrine cell projections through the epithelium (white inset in E) (1.9 µm/section). (F*) Stacked transversal images of F (47 sections, 0.41 µm/section) show membrane-spanning endocrine cells and the location of single image of the same cell shown in D in a middle cross-section of midgut epithelium; the verticle green line in F* corresponds to vertical yellow cross-hair in F.

The *Ae*KR was immunolocalized in the hemolymph side of the rectal papillae's basal membrane (Figure 10). A strong doughnut-shaped fluorescent (red) signal is prominent across the surface of the rectal pad cells projecting out from the rectal wall and which surrounds the valve to the central canal (Figure 10a,b), which will be referred to herein as the outer rectal pad membrane. No signal was seen on the outer rectal pad membrane in tissues incubated with pre-immune serum or pre-absorbed antibodies (Figure 10c,d). As the *Ae*KR would likely be activated by *Aedes* kinin peptides released into the hemolymph this is a plausible location as the receptor would be accessible to its ligand.

While morphological data has been collected on the adult rectum (Hopkins, 1967), only two previous studies explored rectal physiology by immunolocalizing the V-ATPase, Na^+/K^+-ATPase and one exchanger (NHE3) in the rectal pads (Patrick et al., 2006; Kang'ethe et al., 2007). This is the first report, to our knowledge, to identify a neuropeptide receptor in the rectum of an adult mosquito. The location of the *Aedes* kinin receptor on the outer rectal pad membrane suggests it may function to regulate, either directly or indirectly, ion and water reabsorption and perhaps be involved in the rapid epithelial changes in this epithelium observed following a blood meal (Hopkins, 1967). Although *Aedes* kinins stimulate hindgut contractions (Veenstra et al., 1997b), no specific localization of the receptor above background fluorescence could be determined in the muscles. The localization of the *Ae*KR on another novel organ supports that *Aedes* kinins may be integrative signaling peptides.

Figure 10. Confocal analysis of immunolocalization of the *Ae*KR in the rectum, longitudinal view. (A, B) Receptor signal (red) was observed in doughnut-shaped rings of the rectal papillae cell surface (hemolymph side), encircling the valve to the central canal. (C, D) Incubation with pre-immune serum or rabbit antibodies pre-absorbed with their peptide antigen, respectively, resulted in no immunoreactivity on the outer rectal pad epithelium. (A1, B1, C1, D1) Outer-most image of rectum showing circular muscle fibers partially covering the outer rectal pad epithelium. (A2, B2, C2, D2) Single images just under muscles showing the outer rectal papillae cell surface, immunoreactive in A and B. (A3, B3, C3, D3) Single cross-section image through the middle of the rectum showing signal only on the outer epitheium of the rectal papillae in A and B; white circle in C3 outlines a rectal pad with no signal. White arrows point to one outer rectal pad epithelium in each frame. Insert in A1 of immunoreactive stellate cell serves as an internal positive control for *Ae*KR. All images are from 1.53 μm optical sections; the top of the pictured recums connects to the ileum and the bottom leads to the anus (Figure 2).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CHAPTER IV

OBJECTIVE 3:

RNA INTERFERENCE (RNAI) MEDIATED *AE*KR KNOCKDOWN

4.1 Introduction

Protein function can be studied by observing phenotypic changes after knocking down the respective gene expression and thereby eliminating or decreasing the amount of protein present. An established technique for transcript knockdown is RNAi. This technique has been successfully used in *Ae. aegypti*, but is noted to be tissue and gene target specific for its efficacy with variable standards for methods protocols (Boisson et al., 2006).

While it has been known since the 1980s that RNA can interfere with gene expression, it was not until 1998 that Fire et al. (1998), discovered dsRNA as a strong trigger for the pathway. Since this discovery in the nematode *Caenorhabditis elegans*, the mechanisms and further applications of the termed "RNA interference" phenomenon have been studied in numerous organisms. Briefly, in the initiation step, long dsRNA is cleaved into 21-23 nucleotide double-stranded small interfering RNAs (siRNAs) by Dicer, an RNase-III-like dsRNA-specific ribonuclease (Figure 11) (Cerutti et al., 2003). Next, in the effector step, the siRNAs are incorporated into an RNA-induced silencing complex (RISC), comprised of multiple proteins. In the complex, the two siRNA strands are unwound and separated; this is the activation step. The complex, now "activated",

finds and binds mRNAs complementary to the siRNA, after which the mRNA is degraded by an RNase and not translated (Mello and Conte Jr. 2004).

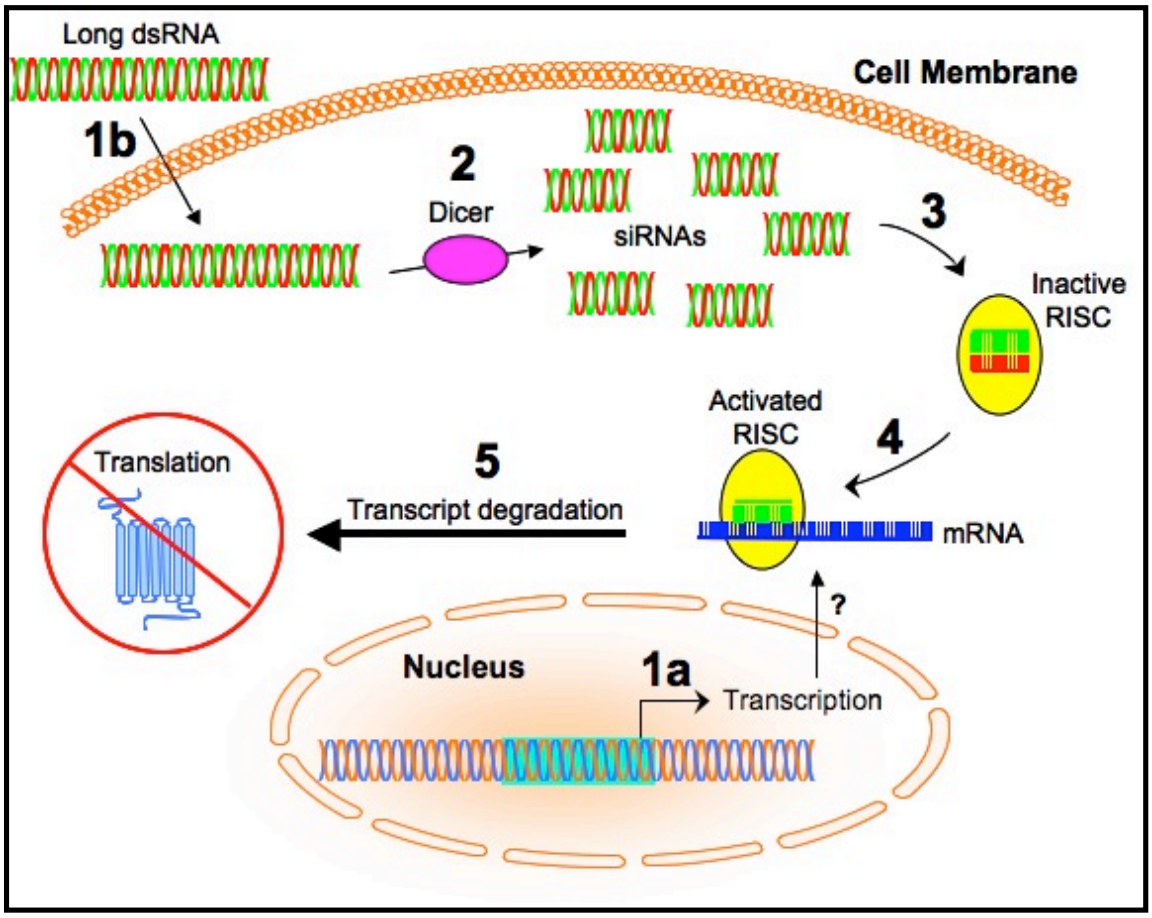

Figure 11. RNAi mechanism. (1a) Target genes are normally transcribed. (1b) dsRNA is transported into cells. (2) Initiation step, (3) Effector step, (4) Activation step, (5) RISC binds mRNA complementary to the siRNA and the transcript is tagged for degradation (modified after Fire et al., 1998; Cerutti, 2003; Mello and Conte, Jr., 2004).

This objective aimed to study the contribution of the *Ae*KR to fluid excretion post blood feeding using RNAi-mediated transcript knockdown and observing phenotypic changes in fluid excretion rate and volume. Knockdown success levels were determined by qPCR and immunohistochemistry. Additionally, the influence of the

RNAi knockdown on fluid excretion rates and overall volume excreted was measured in a precision humidity chamber.

Hypothesis: Phenotypic analysis of RNAi induced *Ae*KR knockdown, accomplished by mosquito injection with dsRNA, will show a decreased fluid excretion rate and decreased total volume of fluid excreted during post prandial diuresis.

4.2 Materials and methods

dsRNA synthesis. Template DNA for dsRNA synthesis was prepared using an *Ae. aegypti Ae*KR cDNA clone (AY596453) (Pietrantonio et al., 2005) with primers Aa-KRdsRNA-T7F (5'–TAATACGACTCACTATAGGGACCGAAGTGGATTTCAAGTG GTTGGAGGTG-3') and Aa-KRdsRNA-T7R (5'TAATACGACTCACTATAGGGCAT CGCTGCCGTTCAGTGTATTGTTGTTTGC–3') with T7 regions (underlined), and amplified by PCR to target a 548 bp region (nucleotides 354–901, Appendix C). MEGAscript®RNAi Kit (Ambion, Austin, TX, USA) was used for dsRNA synthesis per the company protocol with the following modifications. The transcription reaction was be assembled with 2 μg template DNA and incubated at 37°C overnight. The reaction was then incubated at 75°C for 5 min then placed into a 70°C water bath, which was allowed to cool to room temperature. After treatment with DNaseI and RNase the dsRNA was purified, washed, and recovered in hot Elution Solution (95°C). Recovered dsRNA was then de-salted and concentrated using a Microcon column (Milipore,

Billerica, MA, USA) per the manufacturer's protocol. All dsRNA was diluted in nuclease-free water to a final concentration of 8 μg/μl.

Injections. Femtotip Injection Needles (Fisher Scientific, Waltham, MA, USA) were siliconized using SIGMACOTE® (Sigma-Aldrich, St. Louis, MO, USA) by pipetting the solution through the needles then washing with nuclease-free water. Injections were completed using an Eppendorf FemtoJet® MicroInjector (Eppendorf, Hauppauge, NY, USA) set to p_i 100-200 hPa, t_i 0.1 sec, and p_c 0-15 hPa equipped with an Eppendorf TransferMan NK Micromanipulator. dsRNA (2 μg) was injected into the thorax of cold anesthetized female mosquitoes 24 hr PE as previously shown (*Luna et al.,* 2007). Mosquitoes were allowed to recover overnight before male *Ae. aegypti* were added for mating.

Quantitative PCR. Female mosquitoes (n= 50-60) were dissected five days post injection and M. tubules, hindguts, and stomachs were stored in RNAlater Solution (Ambion). Tissues were transferred to TRIzol (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted per the manufacturer's protocol. Extracted RNA was treated with DNase I (Invitrogen), and then the TRIzol protocol was repeated to remove proteins, enzymes, and degraded DNA from the DNase I treatment. Total RNA was solubilized in 15 μl nuclease-free water with 1 μl RNaseOUT (Invitrogen). cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen) using ~3 μg total

RNA as template and random hexamer primers for a final volume of 20 µl per cDNA reaction.

Primers for qPCR were designed using the Primer Express® Software Version 3.0 (Applied Biosystems, Carlsbad, California, USA). The *Ae*KR amplicon, a 59 bp amplicon (nucleotides 1340-1398) at the end of the predicted fourth transmembrane region and spanning the exons 2-3 boundary of the *Ae*KR (Appendix C), was amplified with primers AaKR_qPCR_F (5'–TGGCCGCTCCAACTCTGT–3') and *Aa*KR_qPCR_R (5'–TGTCAATCGTTAGCCTG GGC–3'). BLAST search of this region resolved no similarity to other regions of the *Ae. aegypti* genome. The 18S rRNA gene was amplified using primers 18S_rRNA_(forward) (5'–CCTTCAACAAG GATCAAGTGG–3') and _rRNA_(reverse) (5'–GGAGTAGCACCCGTGTTGG–3') per (Sanders et al., 2003) to normalize experiments. Primer and cDNA concentrations were optimized; 0.5 µl cDNA were analyzed in each well with 1 µl of 5 µM AaKR_qPCR_F and 3 µl of 5 µM *Aa*KR_qPCR_R or 3 µl of 5 µM 18S_rRNA_(forward) and 1 µl of 5 µM 18s_rRNA_(reverse). Quantitative PCR was performed by an ABI-7300 (Applied Biosystems) using POWER/SYBR® Green Master Mix (Applied Biosystems). The comparative threshold cycle (C_T) method was used to assess transcript levels and construct a Gene Expression Plot. Results of four independent replicates were analyzed by t-test in PASW Statistics 18 (SPSS Inc., Somers, NY, USA) to a 95% confidence interval (CI).

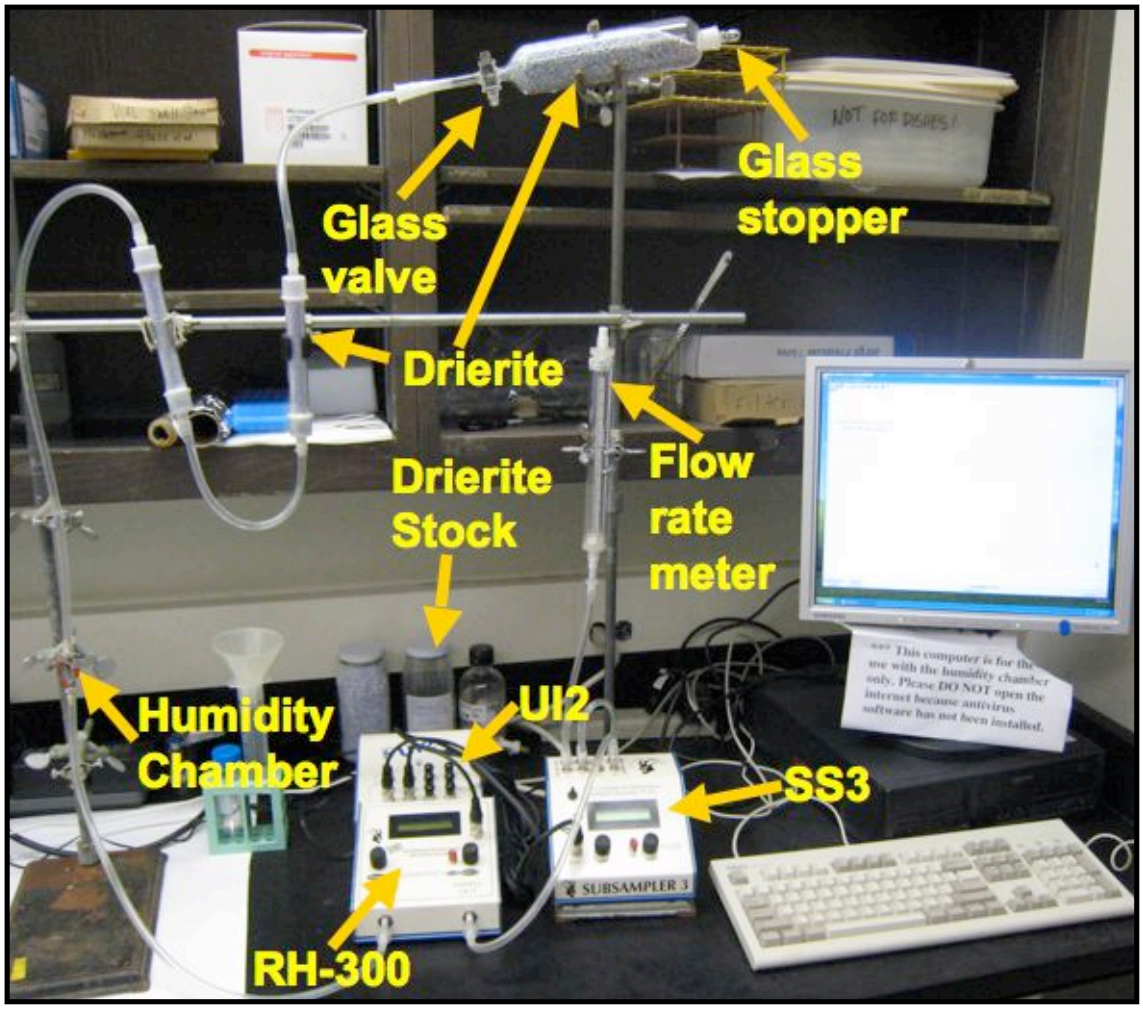

Figure 12. Humidity chamber set up. Turn on the UI2, RH-300, SS3, computer, and monitor. Remove the glass stopper and turn the glass valve 90 degrees to open it. After allowing the pump to dry the chamber and systems air, place a blood-fed female in the humidity chamber and begin recording the humidity using the Sable Systems Software.

*Ae*KR RNAi evaluation by *in vivo* fluid excretion assay. Injected females, 5-9 d post-treatment with either the *Ae*KR dsRNA or controls (EGFP dsRNA or nuclease-free water), were blood fed one-at-a-time then placed into a precision humidity chamber with constant flow dry air (100 ml/min) to measure excretion as previously described (Figure 12) (Coast, 2004). Humidity readings for the chamber were recorded every 0.01 s for 60 m by an RH-300 flow-through humidity analyzer (Sable Systems, Henderson, NV, USA) at room temperature. The system was calibrated by applying known volumes of distilled water (0.5-1.5 μl) to the chamber (Gibbs et al., 1997). Results were analyzed in Expedata (Sable Systems) and then by Repeat Measures ANOVA to a 95% CI.

4.3 Results and discussion

In the M. tubules and hindgut, *Ae*KR dsRNA treatment resulted in a 68.5% and 60.25% decrease in transcript abundance, respectively (Figure 13b,c), and significant protein knockdown, as demonstrated by immunohistochemical staining analysis under identical microscope settings (Figure 13d-g). In the rectum the fluorescent signal was saturated in controls and faint in the knockdown mosquitoes. Protein knockdown in ileal and rectal muscles was inconclusive due to background staining. The dsRNA treatment had a lesser effect in the stomach (22% decrease in transcript abundance), thus, observed fluid secretion RNAi phenotypes are likely associated with decreased *Ae*KR abundance in the M. tubules and the rectum.

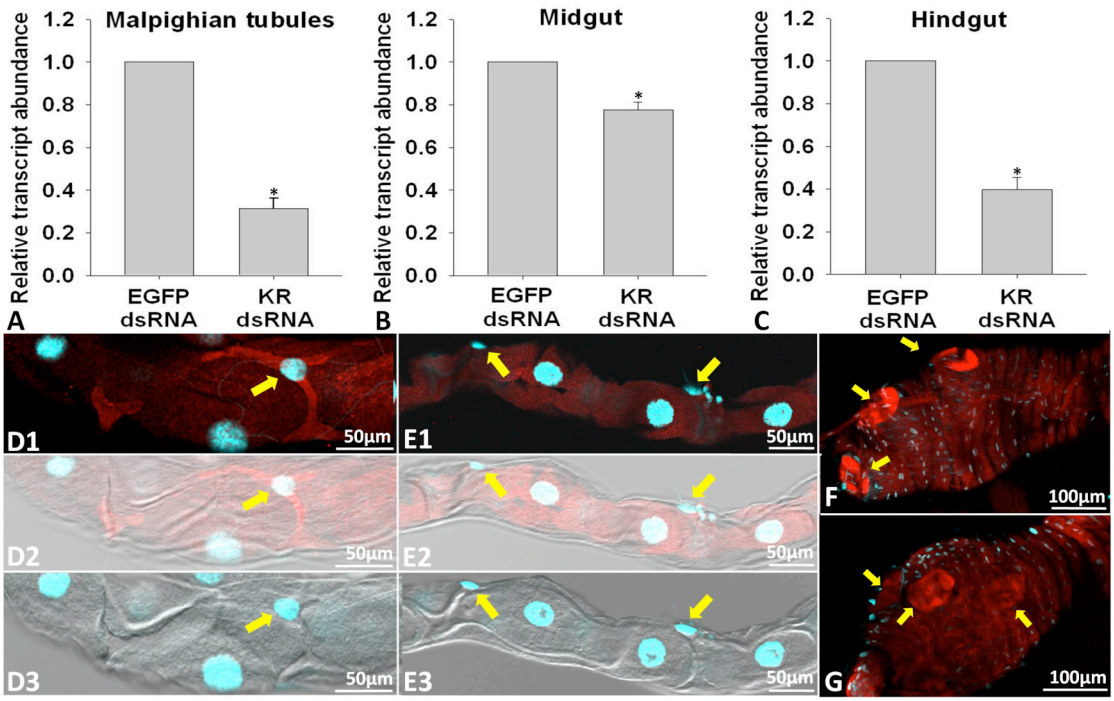

Figure 13. RNAi induced *Ae*KR knockdown. (A-C) In RNAi knockdown females qPCR showed significant decreases in *Ae*KR relative transcript abundance in the M. tubules, stomach, and hindgut, compared to controls. (D-G) Immunohistochemistrical staining (red, *Ae*KR; blue, cell nuclei) confirmed protein knockdown, showing significantly diminished fluorescence in *Ae*KR dsRNA treated females (E,G) compared to controls (D,F) (1.53 µm optical sections). (B2,C2) Merged fluorescence and light microscopy images show *Ae*KR signal in stellate cells. (B3, C3) DIC image confirms stellate cell location. Yellow arrows indicate stellate cell nuclei (D,E) and outer rectal pad membranes (F,G).

In mosquitoes, three phases of postprandial diuresis can be distinguished: peak, post-peak, and late (Bradely, 1987; Williams et al., 1983). These phases last 15, 45, and 60 minutes, respectively, totaling 2 hours (Beyenbach, 2003a). During the first hour females excrete nearly 40% of the water, sodium, and chloride from the ingested blood meal (Williams et al., 1983). Although these three phases operate on a continuum, distinguishing them allows analyzing potentially sequential hormonal regulation.

In *Ae*KR silenced females, a significant decrease was observed in their excretion rate from 5-30 minutes post blood feeding (Figure 14a). Accordingly, the total urine volume excreted decreased, after a slight delay, with significant differences between the *Ae*KR dsRNA treated females and the controls detected at 25 min post blood meal (Figure 14b). These data suggest that under normal conditions the *Ae*KR is activated during the peak phase, and continually stimulated through the beginning of the post-peak phase. The cumulative effect of *Ae*KR silencing resulted in a nearly 50% decrease in the total urine volume excreted by silenced females during the post-peak phase (Figure 14b). While previous work has highlighted the stimulatory effects of *Aedes* kinins on isolated M. tubules, this is the first study showing their contribution to fluid excretion *in vivo*.

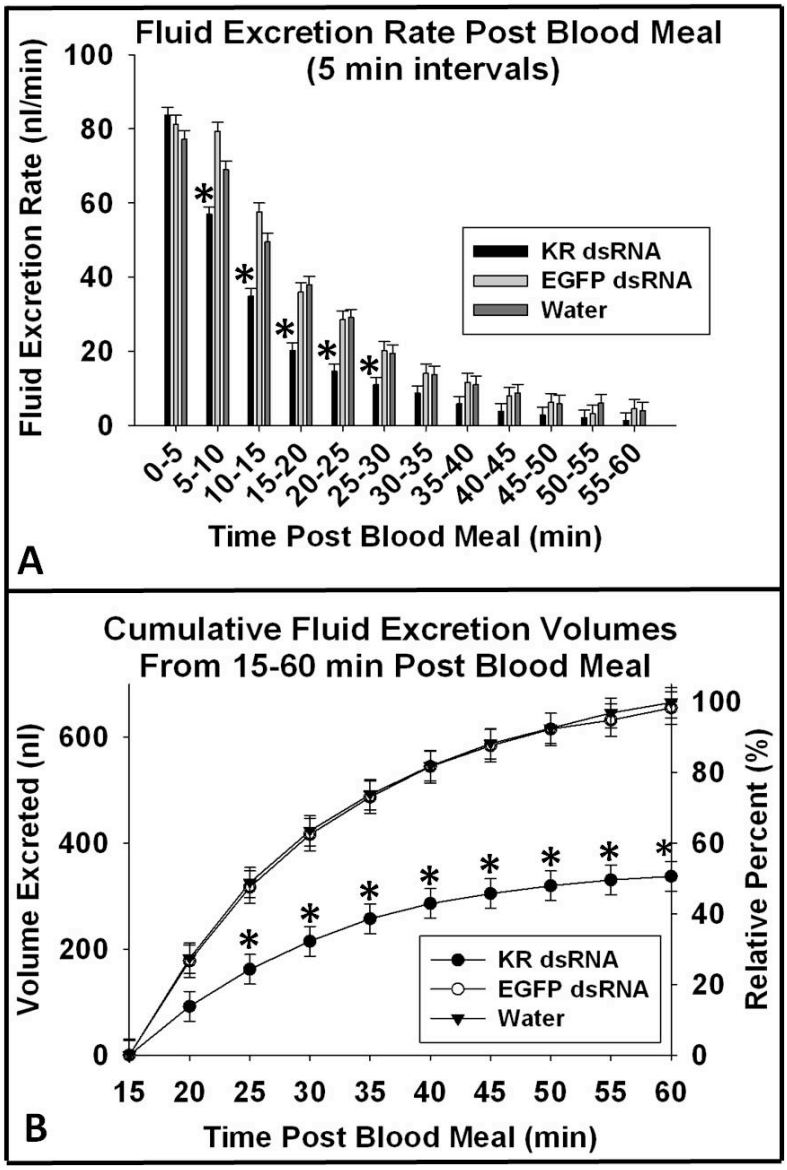

Figure 14. RNAi induced *Ae*KR knockdown associated phenotype. (A) Fluid excretion rates in *Ae*KR dsRNA treated females compared to controls was significantly reduced during the period from 5-30 min post feeding. (B) The total volume excreted was significantly lower in knockdown females than in controls from 20-60 min post blood meal. A 50% decrease in total volume excreted was observed from 15-60 min post blood meal. (* = $p<0.01$)

CHAPTER IV

CONCLUSIONS

This is the first report identifying the *Ae*KR in the head, posterior midgut, ovaries, and hindgut of any mosquito species. Specifically, the *Ae*KR was immunolocalized in the endocrine cells of the posterior midgut and in the membrane encircling the valve to the central canal of the rectal papillae. These findings suggest that *Aedes* kinin peptides function in organs other than the M. tubules, where studies assessing their function have primarily focused. These findings highlight the strong possibility that *Aedes* kinins act as integrative signaling hormones, rather than just diuretic hormones. Further, this is the first report assessing the function of the receptor to fluid excretion in the whole, live mosquito. This study has shown the pronounced importance of the *Ae*KR in the M. tubules and hindgut to proper rapid post prandial diuresis.

Not only do the results from this study confirm the presence of the *Ae*KR in novel locations for any mosquito species, but they highlight the broader potential role of *Aedes* kinins in mosquito homeostasis and possibly their role in the evolutionary success of the mosquito blood feeding adaptation. As successful blood feeding relies on appropriate mosquito behavior, i.e. stopping feeding when fully engorged which is controlled by signaling in the brain, followed by effective digestion of the meal in the stomach to retain nutrients for ovary development and reproduction while rapidly voiding excess ions and fluid through the M. tubules and hindgut, it is noteworthy that

the *Ae*KR has now been identified in all of the organs central to these functions: the brain, stomach, M. tubules, rectum, and ovaries. Improving our understanding physiological components essential for successful blood feeding may lead to novel methods to inhibit this behavior and its related process, which may ultimately be employable for future vector control.

Further understanding the function of the *Ae*KR in the posterior midgut and in the rectum as well as working to localize the receptor in the ovaries is an exciting direction for future research. While this study has laid the groundwork to begin to unravel the story of the *Ae*KR as a GPCR involved in integrative endocrine signaling that may be essential for successful blood feeding and physiological changes in the female immediately post-feeding, much is still unknown about its function. Further, these finding emphasize that many of the well-known ‘diuretic hormones’ may have other undiscovered functions in the mosquito.

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APPENDIX A

Bovine Blood Collection and Defibrination Protocol.

Purpose:

Defibrinated blood is blood without fibrin, a protein involved in the process of blood clotting. Without fibrin blood can be stored at 4^{o}C for later use without it clotting. There are alternate methods to keep blood from clotting, such as adding anticoagulants to fresh blood samples. However, because our research aims to study physiological processes in the mosquito we cannot use anticoagulants because they could possibility alter normal mosquito physiology. Thus, defibrination is the best option as it provides a blood meal for female mosquitoes that is similar to natural vertebrate blood. Defibrinated blood can also be purchased from HemoStat laboratories (www.HemoStat.com), where 30 ml of defibrinated rabbit blood costs $19 and the shipping is $28 (part #DRB030). Combined, the cost to purchase 30mL of blood from this company is $47. This company ships the blood overnight, so it is an excellent resource if blood is needed quickly and there are no slaughters scheduled at Rosenthal Center. This simple protocol is a way to obtain at least 500 ml of defibrinated bovine blood for research purposes, such as blood feeding female mosquitoes, for no cost.

Procedure:

a. Before starting, prepare the work area where the blood will be defibrinated.
b. Autoclave:
    i. 50-100 glass marbles
    ii. Large 2000 ml flask
    iii. 2 x 1000 ml beakers
    iv. 2 x 500 ml beakers
c. Prepare work area:
    i. Clean and remove excess materials from the hood that will be used. There should be nothing else in the hood before beginning the protocol.
    ii. Place at least 3 empty biohazard bags next to the hood.
    iii. Place large syringes (2-3 is usually enough), plastic bulbs, or large pipettes (the glass ones that can hold 10-20 ml) in the cleaned hood. These will be used to collect the liquid defibrinated blood from around the blood clot that will form in the large flask.
    iv. Place a small shaker in the hood on a low setting (you can turn it ON when you return later with the blood). Cover the shaker with foil to ensure that it is not contaminated with blood should there be a spill.
    v. Place approximately 10 of the autoclaved marbles into one of the autoclaved 500 ml flasks and place it onto the shaker (which is OFF).
    vi. Have the desired number of 50 ml plastic tubes close by. These tubes will later be used when the defibrinated blood is aliquotted at the end of the procedure. Example: if you plan to collect 500 ml of blood you will need 10 of these tubes and each will be filled with 50 ml. Label the tubes with the type of blood (bovine if the

collection will be during a beef slaughter), the date of collection, and the vial number with a blank spot after it that you will fill in later (this is to distinguish where the blood from that specific tube come from, for example the top of the 500 ml flask or the bottom. The blood on near the top of the beaker containing the defibrinated blood will have fewer clots and be the best for feeding. It is therefore helpful to be able to distinguish these higher quality samples at a later time when it is time to use the blood for feeding).

2. Collect blood during beef slaughter at Rosenthal Center:
    a. Contact the staff at Rosenthal to determine their scheduled slaughters dates and request permission to collect blood on the preferred date.
    b. Bring a signed letter of intent from your PI stating that the blood will be used only for research purposes and not for human consumption.
    c. Bring the large 2000 ml flask (more than one can be brought if more blood is needed because there is plenty of blood from one cow to fill several, but more than 2000 ml will not likely be needed to maintain a mosquito colony as it expires within three weeks of collection. It is recommended to bring two to have one extra beaker if there are errors during the defibrination process. Further, if after returning to Heep Center it is determined that more blood will be required it is possible to return to Rosenthal Center that same day as the slaughter often last several hours.
    d. Place 50-100 marbles in the collection flask before leaving Heep Center (leave 10-20 autoclaved marbles in the hood for defibrination).
    e. Check in with the Rosenthal Center staff upon arrival for directions on where to put on a hair net, hard hat, and white lab coat, which are required for entering the slaughter room.
    f. Once dressed, enter the refrigerated slaughter room give the collection flask(s) to the staff, they will know what to do with them.

g. After they have filled with flasks with blood take them back to the lab in Heep Center. To do this: bring a large container with a lid that is big enough to carry all of the flasks that are filled with blood (styrofoam boxes and coolers work well). Keep the blood covered while waking back to keep the blood out of sight of other people on campus.
h. After the Rosenthal Center staff has collected the blood, continually and slowly agitate the vials from the time you are given the blood (in Rosenthal Center) until returning to lab. This motion keeps the blood flowing which helps clots form around the marbles.
i. Make sure to bring the following when leaving Heep Center to go collect the blood in Rosenthal Center: gloves (each person going to collect blood will need two pairs: one for during the blood collection, and a pair to change into before walking back to Heep), large Styrofoam container(s) (or cooler) with lid(s) to place the beaker(s) of blood in they are transported back to Heep Center (keep the blood out of sight and ensure there is no blood on the outside of these containers), and paper towels to clean up messes and spills and wipe off the outside of the containers before walking back to the lab.

3. Defibrinate the blood:
    a. Place the containers holding the flasks of blood into a large cart with wheels and have a second person slowly rock the cart back and forth to keep the blood moving (agitate it).
    b. Change into clean gloves. Place used gloves and any paper towels, gloves etc. used while at Rosenthal Center into one of the biohazard bags. Place all disposable material used during the defibrination procedure in this bag and autoclave it when finished.

c. Approximately 20 minutes after the blood was originally placed into the flask begin separating the defibrinated blood from the clot.
    i. During this time period the fibrin should clot around the glass marbles leaving behind liquid blood that is devoid of fibrin, and thus will not clot while being stored or when prepared to feed mosquitoes. The MAJORITY of the blood will clot and leave only a small amount of liquid defibrinated blood relative to the original total volume. This is normal; there will still be more than enough liquid blood left to collect.
d. Take the large flask from the cart and pour any easily separated liquid blood into one of the autoclaved 1000 ml beakers.
    i. This requires two people. Have one person lift/tilt the beaker and pour the liquid blood into the clean 1000 ml beaker and the other should use anything that is sterile, hard, and long (such as a 25 ml automatic pipette tip, for example) to push and hold the clot out of the way of the beaker opening so that the liquid un-clotted blood can flow past the clot into the other beaker.
e. When no more blood can be poured from the collection beaker into the 1000 ml beaker, pour the liquid blood from the 1000 ml beaker into the 500 ml flask containing about 10 glass marbles on the small shaker and turn the shaker on a low setting. You need this step to remove small clots that were poured out with the liquid defibrinated blood in step 2-d-I (above).
f. Next, collect the remaining liquid blood from around the large, main clot in the collecting flask using a large pipette, plastic bulbs, or syringes and transfer it to the 500 ml flask on the shaker.
g. Allow the blood to be agitated in the 500 ml flask for at approximately 10 minutes once you have finished separating the defibrinated blood from the clot.

h. Remove the flask from the shaker and aliquot 45-50 ml into the plastic tubes you labeled before starting.
    i. Take the first 50 ml aliquot from the top layer of the 500 ml flask and label this vial #1. This blood should be least likely to contain any clots and thus should be the best quality blood. Continue this process of collecting the top layer of blood until you reach the bottom of the 500 ml flask. Label them in the order the blood was removed (ex: 1, 2, 3… etc.).

4. Store the aliquoted defibrinated blood in the 4^{o}C fridge in the main lab.
    a. Make sure each tube is clearly labeled and dated.
    b. Smaller aliquots can be made instead, if desired.
5. Thoroughly clean the work area in the hood area and wash and then serialize all used glassware and marbles.
    a. Place the left over blood and clots in one of the empty biohazard bags and tie it shut. Place this bag in a second, empty, biohazard bag in case it breaks and tie that one shut as well. Autoclave the bags to sterilized the blood, and then properly dispose of them.
    b. Clean the hood.
    c. Clean any blood spills on the floor etc.
    d. Wash the shaker if any blood got on it.

APPENDIX B

Artificial Blood Feeding System Set-Up and Protocol.

Purpose:

To feed female *Ae. aegypti* in the laboratory a series of three glass feeders were set up in a series. Defibrinated blood was then fed to females through these artificial membrane feeders as detailed below. This protocol allows for feeding individual insects or more than 100 at a time for colony maintenance.

Procedure:

I. General set-up:
   a. Materials:
      i. Glass feeders
         A. *Ordered from:*
         *Chemglass Life Sciences *3800 N. Mill Rd., Vineland, NJ
         *Phone: 1-800-843-179 *Fax: 1-800-922-4361
         *Part description: Custom Glass Jacketed Membrane Feeders, 18mm OD Beaded Top, 6mm OD Inner Tube, 14mm OD Tube, 4mm OD side arms, 31.5mm OAH
         *Ordered 5 feeders at $98.50 each (total = $492.00, no shipping) on November 4, 2009.
         B. *Other option:* contact the glass blower from the Chemistry Department on campus and he can make them. He estimates $40-50 for the small feeders and variable price based on the size of feeder desired.
         *Contact: Bill Merka, Research Instrumentation Specialist

*Phone: 845-2735 *email:merka@mail.chem.tamu.edu

ii. Blood

A. Ordered from:

*Hemostat Laboratories *Location: Dixon, CA

*Phone: 1-800-572-6888 *Fax: 1-707-678-1150

*Order: Defibrinated rabbit blood, Part no. DRB030 (for 30 mL), price = $19.00, shipping charge $28.00 (will only ship overnight), total cost $47.00

B. *Other option:* collect blood during slaughter at Rosenthal on campus then follow procedure for blood defibrination (Appendix A). This is a free alternative and takes approximately 3-4 hours to complete and clean up.

*Contact: Ray Riley *e-mail: r-riley@tamu.edu

*Other important information: You will need to bring a signed letter from Dr. Pietrantonio stating the blood is for research purposes only and not human consumption, 2 sets of latex gloves, beakers to collect the blood in, and containers (large boxes or Styrofoam containers work) to carry the beakers back in so other people cannot see the blood.

iii. PVC tubing (to connect the feeders to eachother, the pump, and beaker with warm water)

*Purchased from: BioBio store on campus

*price: $0.45 per foot *Dimensions: tubing 3/32 x 1/32″

iv. ATP: Adenosine 5'-triphosphate (ATP) disodium salt hydrate

*Sigma *Product no.: A1852-1VL

*Price: $36.00 per vial *Amount: 30 mg per vial

v. Pump (for water circulation)
*"VWR Pump, Variable Flow" *Cat. no. 54856-075
*Pump type: medium flow *Flow rate: 0.4 to 85 mL/min
*Price: $227.88 each

vi. Hot plate, thermometer, and large beaker to heat the water in that will be pumped through the outer jacket of the glass feeders.

II. Pre-feeding preparation:

a. The morning that mosquitoes will be fed add 1.67 mg ATP for every 1 ml blood.
   i. Preparing 1 ml at time by combining 1 ml blood with 1.67 mg ATP is the preferred method because the ATP will degrade and the new ATP + blood will need to be prepared within approximately 6 hours.
   ii. After ATP is added to blood aliquot, keep the blood at 4^{o}C except when loading it into feeders. The ATP degrades slower at this temperature than at room temperature.
b. Fill the 1000 ml beaker approximately half full with tap water.
c. Warm the water to about 50^{o}C on a hot plate.
   i. Anywhere from approximately 40-60^{o}C works, but 50^{o}C is optimal (the mosquitoes will feed on the blood when a water bath temperature in kept this range).
d. Cut a piece of parafilm about 2 cm wide (Figure 7-1).
e. Very carefully stretch the parafilm width-wise about 2.5 to 3 times its initial width (Figure 7-2). Stretching the parafilm length-wise does not work as well because it will be too thin and break or "melt" once it contacts with the warm blood and water.

i. It typically requires several practice rounds to learn to properly stretch the parafilm properly. The thickness of the parafilm is a very important variable. If the film is too thin it will break when it touches warm blood and if it is too thick the mosquitoes will not be able to penetrate it and obtain a blood meal.

f. Cover the bottom of the blood feeders with the parafilm (Figure 7-3).

g. Smear a thin layer of Vaseline over the parafilm.

h. Turn on the pump and set it anywhere from 5-10 (I set it at 6, a medium speed value). This will drive water through the feeder series to warm the blood to mimic the human body temperature (Figure 5).

i. Add 100-400ul blood with ATP into the feeder by pipetting it into the small hole on the top of the feeder (Figures 7-4). It should run down the inside of the feeder and collect in a shallow pool over the parafilm (Figures 7-5, 7-6).

(Figure 7 repeated from the main text.)

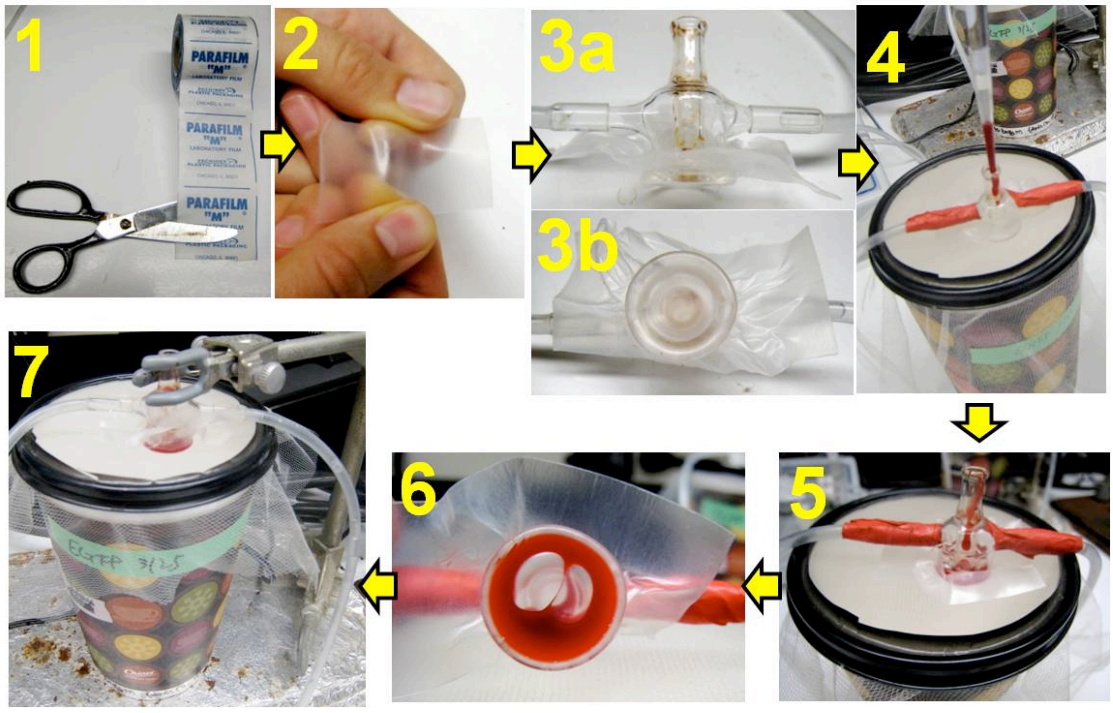


Figure 7. Artificial membrane blood feeders preparation. (1) Parafilm was cut into approximately 2 cm wide strips. (2) Cut parafilm was stretched along its short axis as pictured. (3) The stretched parafilm was applied to the bottom of the blood feeders and adhered to the glass to ensure no blood leakage. A thin layer of Vaseline was coated onto the parafilm after it was stretched over the bottom of the feeder. (4,5) The feeder was placed on the mesh top of a cage containing the female mosquitoes to be fed and blood was pipette (~100-600 µL) into the feeder through a thin tube opening on the top. (6) The blood was visualized from the bottom to ensure there was a working seal around the rim and there were no holes in the membrane. (7) The feeder, equipped with blood was placed on top of a cage, which allowed female(s) access to the meal.

(Figure 5 repeated from the main text.)

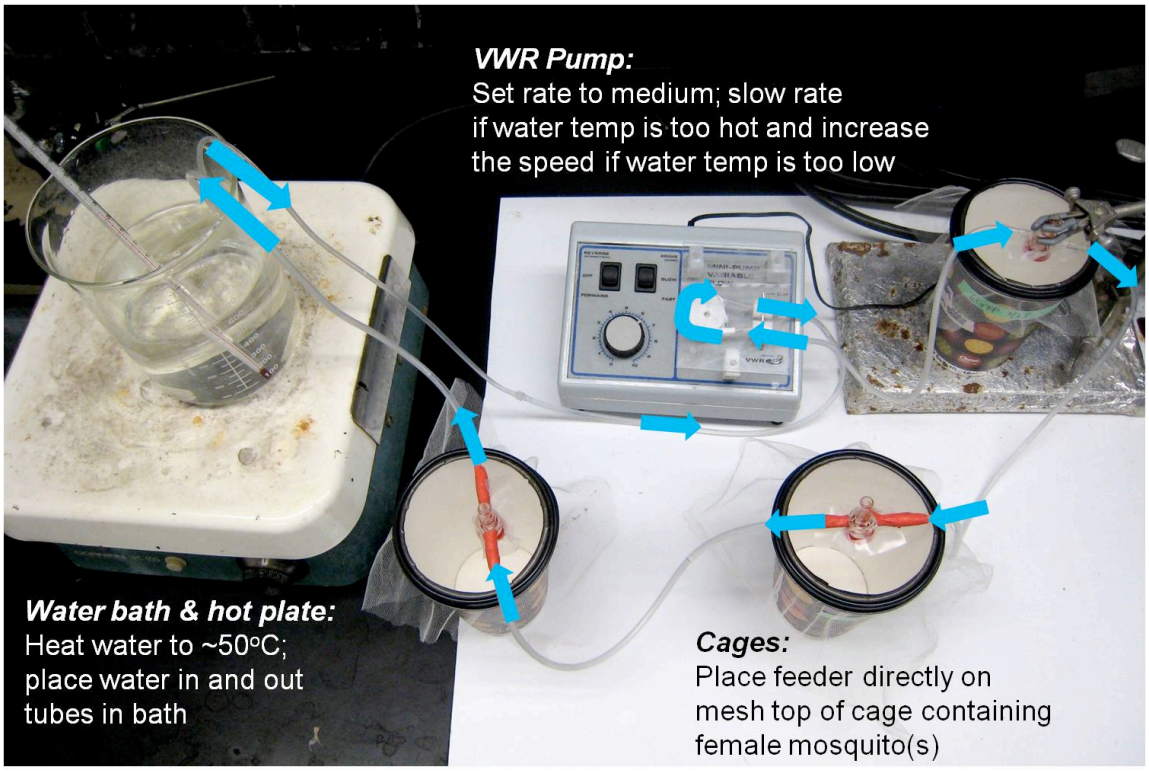


Figure 5. Water flow through the artificial blood feeding system set-up. Water in a 1000 ml beaker was heated to approximately 50°C on a hotplate. A variable rate pump drove hot water to leaves the water bath (beaker) through PVC tubing, which then circulated through the pump apparatus and into the first feeder. Water in the first feeder warmed the blood to around 37°C (human body temperature). Water left the first feeder and repeated the heating process for blood in two subsequent feeders, attached in series. Water, then cooled to close to room temperature was recycled back into the water bath where it was heated to 50°C and the cycle repeated. Blue arrows indicate direction of water flow.

III. Feeding

a. The membrane feeder(s) loaded with blood should be placed directly on the mesh top of a cage containing the female mosquito(s) to be fed (Figures 7-7, 5).

b. While feeding, the mosquitoes should not be disturbed. When disturbed during feeding, female do not feed to completion, and thus the physiological changes initiated after a full blood meal are not likely to occur. This can alter subsequent physiological processes being studied.

c. Allow the female(s) to feed.

    i. Feeding a single female should take at 10-15 minutes, but can happen immediately or never, depending on how well the blood was prepared and how hungry the female is.

    ii. To feed a crowded cage for colony maintenance all three feeders should be placed on the top of one cage containing the females and leave it for 30 minutes to 1 hour. Check the water bath temperature frequently during the hour to maintain it in the desired temperature range.

d. If no females feed within 20 minutes there is likely a problem with the feeders. The following are common problems that can occur with the feeders and troubleshooting techniques for each.

    i. **Problem 1:** The blood is no longer fresh enough or the females cannot penetrate the membrane. **Remedy 1:** Replace the blood and parafilm and try to stretch it thinner. To help distinguish if the problem is the blood or the membrane watch the mosquitoes. They will probe the blood and their proboscis can be observed penetrating the membrane. Should a female's proboscis frequently penetrate the membrane, but then with-draw before feeding (which can be distinguished by observing the changing size and color of her abdomen from small and white/grey to distended and

red), then the problem is probably the blood. Is the female walks over the membrane, stopping frequently and tilting her abdomen so that it is further from the membrane than her head, but is unable to penetrate the membrane, then the parafilm is probably too think.

ii. **Problem 2:** The mosquito attempting to be fed rests on the size or bottom of the container and does not probe the blood. **Remedy 2:** The female may have just fed on dextrose or may be too old or too weak to feed. As these are live insects their behavior is not always predictable. Therefore, use different mosquitoes for the time being and allow the one(s) that would not feed to starve for up to 12 hours to increase their hunger. To help diagnose this problem observe their abdomens. If they are slightly distended and white/grey then the mosquito likely just imbibed a large sugar-water bolus.

iii. **Problem 3:** As described in problem 1, the mosquito will probe the blood and insert her proboscis, but will not feed. **Remedy 3:** First attempt to change the blood as described in remedy 3. If the problem persists then the ATP in the working stock of the blood (loaded with ATP) may have been degraded. Prepare a new stock of blood with fresh ATP.

e. When one of the mosquitoes begins to feed watch her very carefully.

i. The proboscis can be observed entering the membrane and then her abdomen will slowly stretch and turn red from the blood.

ii. DO NOT disturb the mosquito during feeding because this will often cause her to not take a fully blood meal, which will alter the fluid excretion pattern and volume, as well as other physiological changes triggered by the meal that are being investigated.

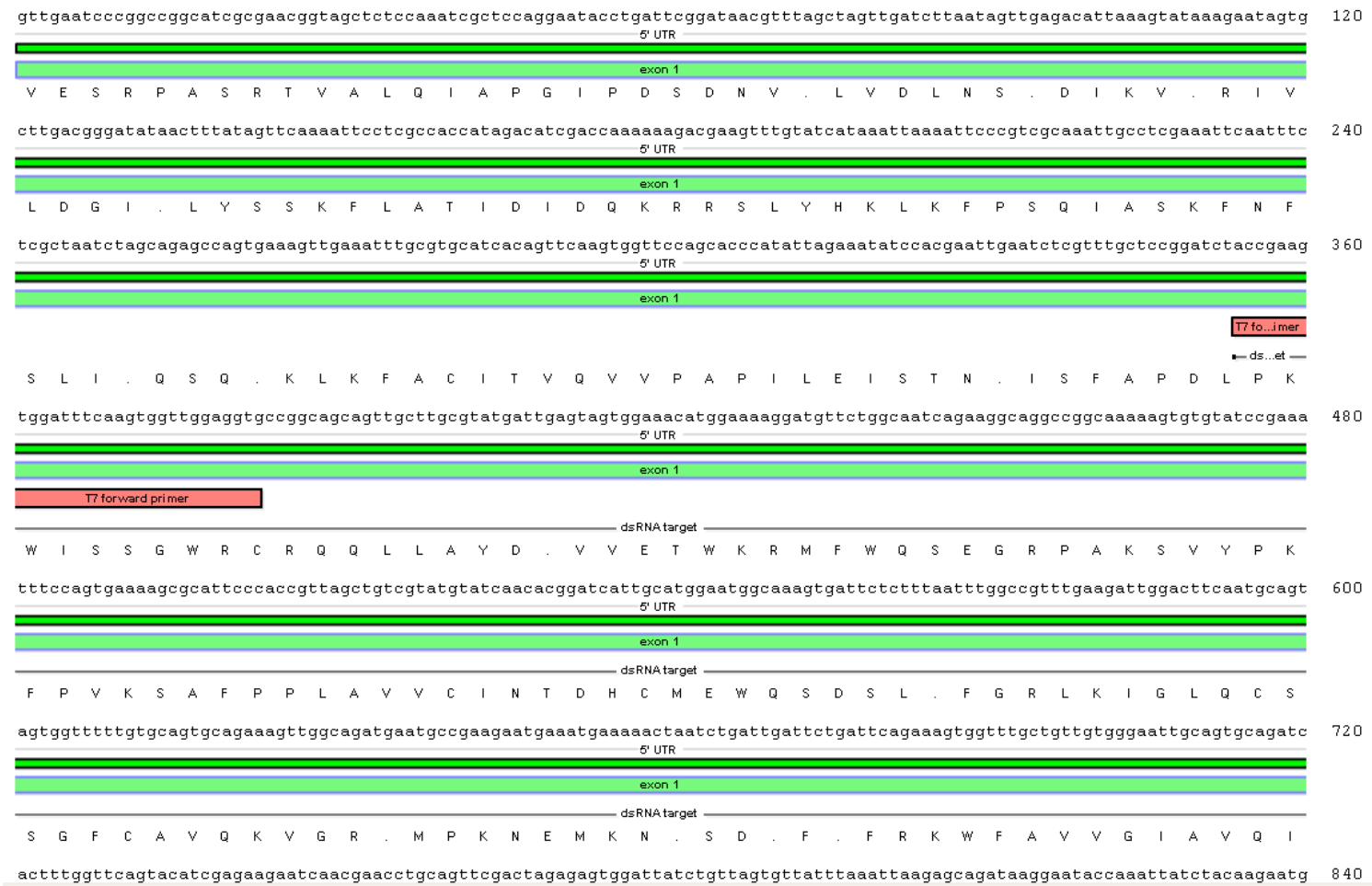
iii. When the mosquito is fully engorged show will slowly retract her proboscis and her abdomen will be significantly larger and red.

iv. At this point, tapping side of the container will likely cause the fully engorged mosquito to fall to the bottom of the container because the large bolus makes it more challenging for the female to fly. Females ingest more than ten times their hemolymph volume in blood when fully engorged.

APPENDIX C

Kinin receptor amino acid and cDNA sequence.

Kinin receptor amino acid and cDNA sequence (GenBank AY596453) showing T7 primers for dsRNA synthesis template, dsRNA template regions, transmembrane regions, 5’ and 3’ untranslated regions (UTRs), the open reading frame (ORF), exon boundaries, qPCR gene specific primers, qPCR amplicon, Poly A tail, and antibody target sites. The predicated residues under the 5’ and 3’ UTRs are only left in this figure to facilitate locating nucleotides in the sequence because these regions do not encode an ORF.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ds...et
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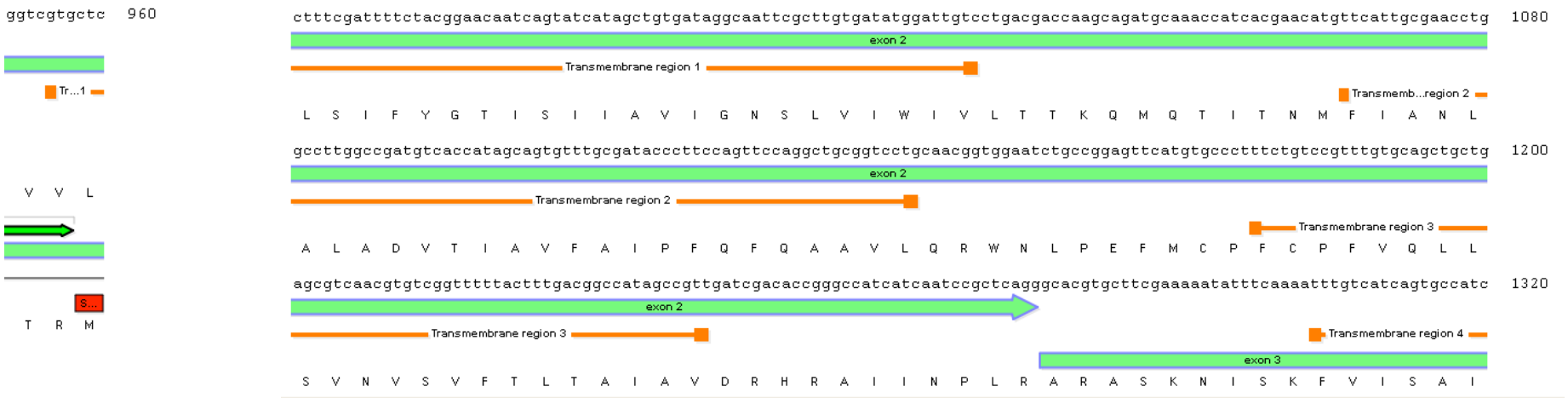
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T R M
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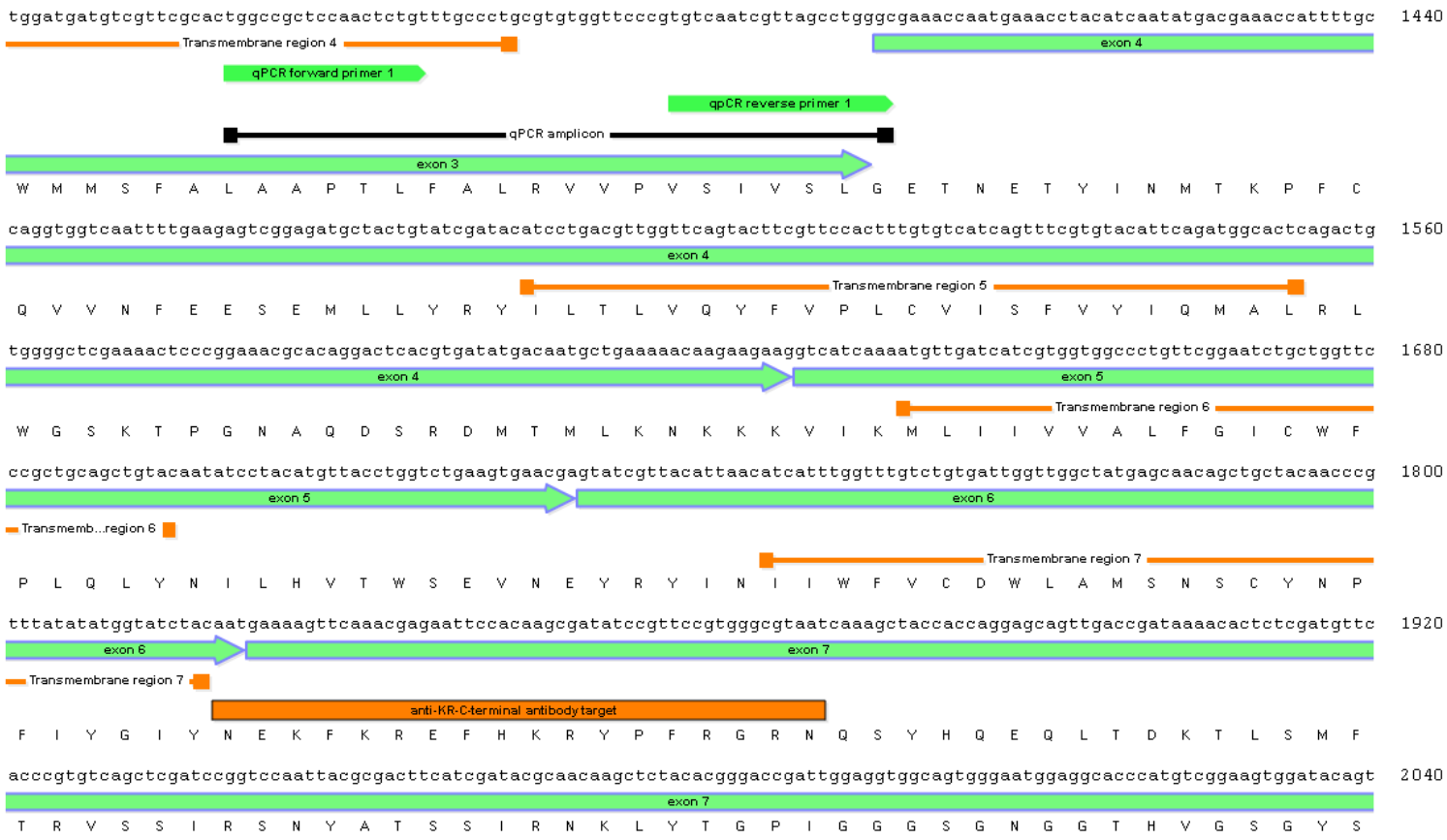
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qPCR reverse primer 1
qPCR amplicon
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exon 5
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exon 6
Transmemb...region 6
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exon 7
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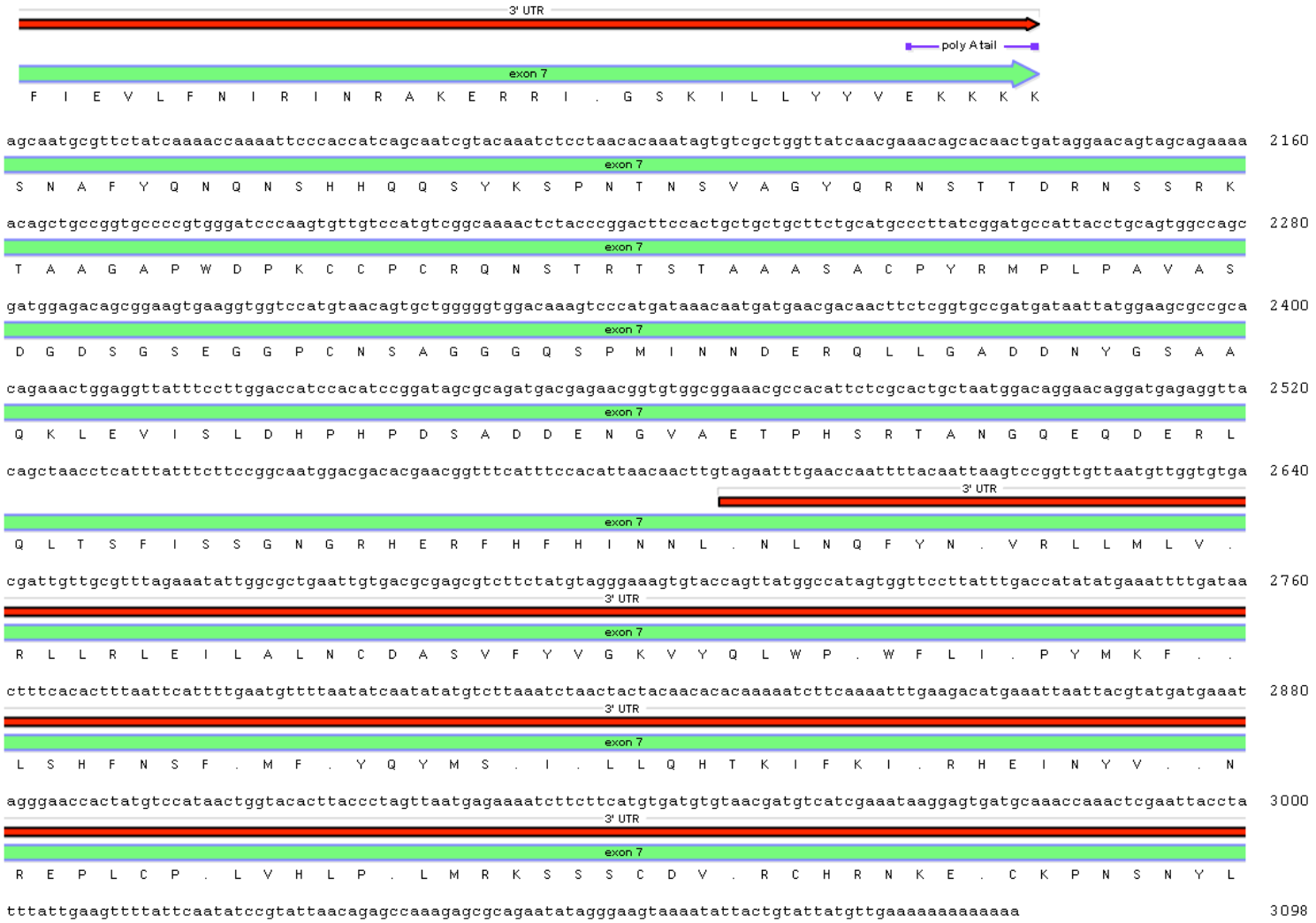
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Selected Peer Reviewed Publications and Formal Research Presentations:

Kersch, C.N., Pietrantonio, P.V., 2011. Mosquito *Aedes aegypti* (L.) leucokinin receptor is critical for *in vivo* fluid excretion post blood feeding and is expressed in novel tissues. (*Under review for FEBS Letters*).

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